University of Alberta

Cross-talk Between Nuclear Receptors in the Transcriptional Regulation of the Genes Encoding Peroxisomal Fatty-Acyl-CoA Oxidase and Enoyl-CoA Hydratase/3-Hydroxyacyl-CoA Dehydrogenase

by

Christopher J. Winrow

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology and Anatomy

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Chris Winrow

Christopher J. Winrow
#205, 2 Ackerman St.
Picton, Ontario
Canada K0H 2T0

Date: JANUARY 26, 1999

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Cross-talk Between Nuclear Receptors in the Transcriptional Regulation of the Genes Encoding Peroxisomal Fatty-Acyl-CoA Oxidase and Enoyl-CoA Hydratase/3-Hydroxyacyl-CoA Dehydrogenase, submitted by Christopher J. Winrow in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Richard A. Rachubinski

Dr. Luis B. Agellon

Dr. Luc Berthiaume

Dr. Tom C. Hobman

Dr. Ellen K. Shibuya

for Dr. Barry M. Forman

Date: JANUARY 26, 1999

ABSTRACT

Peroxisomes are organelles which are responsible for the β-oxidation of long chain fatty acids and the metabolism of hydrogen peroxide. The metabolic activity of peroxisomes can be stimulated by administration of a structurally diverse group of compounds collectively termed peroxisome proliferators. These agents include fibrate family hypolipidemic drugs, antidiabetic thiazolidinediones, eicosanoids, as well as various naturally occurring and synthetic mono- and polyunsaturated fatty acids. The transcription of genes encoding enzymes responsible for peroxisomal β-oxidation is regulated by a group of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs). PPARs form heterodimers with the 9-*cis*-retinoic acid receptor (RXRα) and bind to cognate peroxisome proliferator-response elements upstream of target genes. PPARs govern the regulation of a number of genes involved in lipid metabolism and homeostasis including those encoding the peroxisomal β-oxidation enzymes fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, enzymes which carry out ω-hydroxylation, as well as apolipoproteins AI and CIII and fatty acid binding protein. The focus of my research has been to investigate the interplay between nuclear receptors and auxiliary factors in directing gene expression, with particular attention to the regulation of peroxisomal β-oxidation genes via the peroxisome proliferator signalling pathway. The results of my studies have established the involvement of hepatocyte nuclear factor-4, thyroid hormone receptor α, retinoid-like Z receptor, RXRα and PPARα in both the positive and negative transcriptional regulation of genes encoding core enzymes responsible for fatty acid metabolism. Calreticulin is a Ca^{2+}-binding protein chaperone, suggested to be a general regulator of nuclear receptor activity. The results I have

obtained indicate that although calreticulin interacts with nuclear receptors *in vitro*, it does not have a general effect on all classes of nuclear receptors in a cellular context. My research has demonstrated multilevel control by distinct signalling networks in the regulation of genes involved in peroxisomal β-oxidation of fatty acids, thereby providing suitable targets for pharmacological intervention and contributing to our basic understanding of nuclear receptor signalling and the peroxisome proliferator response.

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LIST OF ABBREVIATIONS

ACTR	activator of the thyroid and retinoic acid receptors
AF	activation function
AOx	fatty acyl-CoA oxidase
AR	androgen receptor
ARE	adipocyte lipid-binding protein response element
BTM	basal transcription machinery
CARLA	co-activator-dependent receptor ligand assay
CBP	CREB-binding protein
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CPS	carbamoyl phosphate synthetase
CREB	cAMP response element-binding protein
CRT	calreticulin
CoA	coenzyme A
CMV	cytomegalovirus
CYP	cytochrome P450
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle's medium
DR*n*	direct repeat of TGACCT-like motifs separted by *n* nucleotide(s)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift analysis
ER	estrogen receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIP-1	GR-interacting protein 1
HD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNF	hepatocyte nuclear factor
LBD	ligand-binding domain
LTB4	leukotriene B_4
luc	luciferase gene
LXR	liver X receptor
MAP kinase	mitogen-activated protein kinase
NCoA	nuclear receptor co-activator
NCoR	nuclear receptor co-repressor
PBP	PPARγ-binding protein
p/CAF	p300/CBP-associated factor
p/CIP	p300/CBP co-integrator associate protein
PGC	PPARγ-co-activator
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-response element
RAC3	receptor-associated co-activator 3

RAR	retinoic acid receptor
RIP-140	receptor-interacting protein 140
ROR	retinoid-like orphan receptor
RXRα	9-*cis* retinoic acid receptor
RZR	retinoid-like Z receptor
SDS	sodium dodecyl sulfate
SMRT	silencing mediator for RARs and TRs
SRC-1	steroid receptor co-activator-1
STAT	signal transducer and activator of transcription
T3	3,3',5-triiodothyronine
TIF-2	transcriptional intermediary factor 2
TRAM	TR-activator molecule
TR	thyroid hormone receptor
TRE	thyroid hormone response element
VDR	vitamin D3 receptor

CHAPTER 1

INTRODUCTION

1.1 Introductory Statement

Peroxisomes are organelles bounded by a single membrane that are responsible for a myriad of cellular functions, including the β-oxidation of fatty acids, the degradation of H_2O_2 and other toxins, gluconeogenesis, as well as cholesterol and bile acid biosynthesis (Bentley et al., 1993; Hiltunen et al., 1996; Mannaerts and Van Veldhoven, 1993; Tolbert, 1981). Over 30 years ago, it was observed that certain agents, later named peroxisome proliferators, could increase both the number and metabolic capacity of peroxisomes (Hess et al., 1965; Paget, 1963; Svoboda and Azarnoff, 1966). Within the past decade, it was determined that novel members of the nuclear receptor superfamily, termed peroxisome proliferator-activated receptors (PPAR), are central mediators of peroxisomal metabolism through transcriptional regulation of genes encoding the core peroxisomal enzymes (Issemann and Green, 1990; Latruffe and Vamecq, 1997; Schoonjans et al., 1996). Substantial roles for PPARs in the regulation of cell growth and development, lipid metabolism and storage, and signal transduction have since been established (for reviews see Desvergne et al., 1998; Reddy and Chu, 1996). PPARs are ligand-modulated transcription factors that direct gene expression through interactions with peroxisome proliferator-response elements (PPRE) located upstream of target genes (Forman et al., 1996; Latruffe and Vamecq, 1997; Schoonjans et al., 1997). We have endeavoured to explore the basic mechanisms by which nuclear receptors cross-talk in order to modulate the transcriptional activity of the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway in response to peroxisome proliferators.

1.2 Peroxisome Proliferators

Peroxisome proliferators were first identified in studies of fibrate drugs, in which it was observed that administration of clofibrate and other hypolipidemic agents caused an induction of peroxisomes in rodent hepatocytes (Hess et al., 1965; Paget, 1963; Svoboda and Azarnoff, 1966). Peroxisome proliferators constitute a class of structurally diverse agents that have marked effects at both the cellular level and in the context of the whole animal. The effects observed in rodents exposed to peroxisome proliferators include hepatomegaly, hepatocarcinogenesis and the induction of genes for both peroxisomal and non-peroxisomal enzymes (Hawkins et al., 1987; Hijikata et al., 1987; Lock et al., 1989; Reddy and Lalwai, 1983; Reddy et al., 1986b). Although chronic exposure to peroxisome proliferators leads to hepatocarcinogenesis in rodents, humans appear to be refractory to the effects of these agents (Bentley et al., 1993; Bieri and Lhuguenot, 1993; Cattley et al., 1998). Peroxisome proliferators are classified as non-genotoxic hepatocarcinogens, as they do not directly interact with, or damage DNA (Shaw and Jones, 1994). Administration of peroxisome proliferators to responsive cells results in a dramatic increase in the absolute number and metabolic activity of peroxisomes (Hawkins et al., 1987; Lazarow and De Duve, 1976; Osumi and Hashimoto, 1978). In particular, the transcription of genes encoding the enzymes responsible for peroxisomal β-oxidation is stimulated (Hijikata et al., 1990; McQuaid et al., 1987; Osumi, 1993; Ozasa et al., 1985; Reddy et al., 1986a; Thangada et al., 1989). Peroxisome proliferators include fibrate hypolipidemic drugs, non-steroidal anti-inflammatory agents, eicosanoids, phthalate ester plasticizers, thiazolidinedione antidiabetic compounds, chlorinated hydrocarbons, as well as naturally occurring and synthetic mono- and

polyunsaturated fatty acids (Forman et al., 1997a; Kliewer et al., 1997; Krey et al., 1997; Lee et al., 1997; Lehmann et al., 1997; Reddy and Chu, 1996; Youssef and Badr, 1998; Zhou and Waxman, 1998). Their actions are mediated by the PPARs (Issemann and Green, 1990). The ability of such structurally diverse compounds to activate PPARs, and the failure to identify directly-interacting PPAR agonists led to hypothesis that peroxisome proliferators are reduced to a common metabolite which activates PPARs.

Recently, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and the thiazolidinedione, BRL49653, have been shown to bind directly to, and to activate the γ isoform of PPAR (Kliewer et al., 1997; Lehmann et al., 1995). Leukotriene B_4 and the hypolipidemic drug, Wy-14,643, have been identified as *bona fide* ligands for the PPARα isoform (Forman et al., 1997a; Kliewer et al., 1997; Krey et al., 1997; Schoonjans et al., 1997). An *in vitro* conformation-based assay that monitors PPAR-DNA binding has shown that specific fatty acids, eicosanoids and fibrate hypolipidemic drugs also act directly as ligands for PPARα and/or PPARδ (Forman et al., 1997a). Additionally, an *in vitro* study employing a high-affinity radiolabelled ligand for PPARα and PPARγ demonstrated the binding of specific mono- and polyunsaturated fatty acids to both these receptors (Kliewer et al., 1997). This study also showed that the eicosanoid 8(*s*)-hydroxyeicosatetraenoic acid binds selectively to PPARα whereas 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 binds directly to PPARγ (Kliewer et al., 1997). Structural analysis has indicated that the large, ligand-binding pocket of PPARs may account for the capacity of these receptors to directly interact with a relatively large number of structurally distinct ligands (Nolte et al., 1998).

Although chronic exposure to peroxisome proliferators leads to hepatocarcinogenesis

in most rodents, exposure to these agents has not been shown to induce hepatomegaly or hepatocarcinogenesis in humans (Bentley et al., 1993; Bieri and Lhuguenot, 1993; Cattley et al., 1998). Guinea pigs are also not susceptible to the tumourogenic effects of peroxisome proliferators (Bell et al., 1998; Bentley et al., 1993; Lake and Gray, 1985). This lack of a carcinogenic response may be due to the reduced level of PPARα found in humans and guinea pigs (Bell et al., 1998; Cattley et al., 1998; Palmer et al., 1998). Guinea pig PPARα is functional, as it has been shown to be capable of inducing transcription from a PPRE-reporter construct in response to peroxisome proliferators (Bell et al., 1998; Tugwood et al., 1998). PPARα is present at a 10-fold lower level in human liver than in responsive species, and human PPARα may contain a splice variant form that decreases its proliferative effects (Palmer et al., 1998).

Several models have been proposed by which peroxisome proliferators may induce hepatocarcinogenesis (Bentley et al., 1993; Christensen et al., 1998; Peters et al., 1997a; Rao and Reddy, 1991). In rodents, the hallmarks of peroxisome proliferators are their ability to increase the levels of fatty acyl-CoA oxidase, peroxisomal fatty acid oxidation and excess production of H_2O_2 and to stimulate cell proliferation and the expression of PPARα (Cattley et al., 1998). The elevation of H_2O_2 without a concomitant rise in H_2O_2-degrading peroxisomal catalase may result in oxidative DNA damage (Rao and Reddy, 1991; Reddy and Rao, 1989; Reddy et al., 1986b). Peroxisome proliferators have also been found to be mitogenic, since they increase DNA synthesis in primary hepatocyte cultures in a glucocorticoid-dependent manner (Plant et al., 1998). It appears that stimulation of the glucocorticoid receptor dramatically increases the transcription of the PPARα gene, and as

a result, makes cells more responsive to peroxisome proliferators (Plant et al., 1998). The recent observation that PPAR inhibits *bcl-2* expression furthers the hypothesis that peroxisome proliferators act as carcinogens by suppressing apoptosis (Christensen et al., 1998; Schulte-Hermann et al., 1991). Direct evidence for the involvement of PPARα in mediating peroxisome proliferator-induced hepatocarcinogenesis comes from studies of transgenic mice in which PPARα has been eliminated. In these studies, 100% of wild-type mice exposed to the peroxisome proliferator Wy-14,643 developed multiple hepatocellular neoplasms, whereas none of the PPARα -/- mice were affected under the same conditions (Peters et al., 1997a).

1.3 Peroxisomal β-Oxidation

Peroxisomal β-oxidation is the main metabolic pathway for the degradation of fatty acids with chain length greater than 18 carbon atoms (*i.e.* C18), typically referred to as very long-chain fatty acids (Mannaerts and van Veldhoven, 1996). This pathway is also employed to metabolize medium and long chain fatty acids with chain lengths between C10-C18, especially in conditions where the capacity of mitochondrial β-oxidation is exceeded (Hashimoto, 1996; Lehninger et al., 1993; Mannaerts and van Veldhoven, 1996; Tolbert, 1981). Peroxisomal β-oxidation is incomplete since fatty acids shorter than C8 cannot be oxidized in peroxisomes. The C8-fatty acids generated by peroxisomal β-oxidation are converted to carnitine esters by peroxisomal carnitine acyl-transferase and directed to the mitochondria for further oxidation (Mannaerts and van Veldhoven, 1996).

The peroxisomal β-oxidation cycle consists of three core enzymes: fatty acyl-CoA

oxidase (AOx), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (HD), and 3-ketoacyl-CoA thiolase. Peroxisomes also contain catalase for the metabolism of H_2O_2 (Hashimoto, 1996; Lehninger et al., 1993). Peroxisomal and mitochondrial β-oxidation of fatty acids both require fatty acyl-CoA as the initial substrate; however, the two forms of β-oxidation are carried out by distinct enzymes and differ in several important respects (see Fig. 1-1) (Eaton et al., 1996; Hashimoto, 1996; Lehninger et al., 1993; Mannaerts and van Veldhoven, 1996). In the first step of peroxisomal β-oxidation, electrons transferred to FAD are directly passed to O_2, yielding H_2O_2, which is then broken down to H_2O and O_2 by peroxisomal catalase. In mitochondria, electrons transferred to FAD proceed through the respiratory chain via the electron-transferring flavoprotein in the mitochondrial inner membrane to yield H_2O and ATP. Another difference between these two forms of fatty acid β-oxidation is that the NADH produced through the action of enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase bifunctional protein is not oxidized within peroxisomes and must be shuttled outside and eventually passed on to the mitochondria, which are capable of oxidizing NADH. Similarly, acetyl-CoA produced in peroxisomes is exported and can be further oxidized by the citric acid cycle in mitochondria. Peroxisomal β-oxidation is preferential for medium-, long- and very long-chain fatty acids (C10-C30), whereas mitochondria carry out the β-oxidation of short-, medium- and long-chain fatty acids (up to C18) (Eaton et al., 1996; Hashimoto, 1996; Lehninger et al., 1993; Mannaerts and van Veldhoven, 1996). In mitochondria, each of the β-oxidation enzymes is a soluble, separate protein; however, the enzymes of the peroxisomal β-oxidation cascade form a multifunctional protein complex (Lehninger et al., 1993). Since plant mitochondria do not perform β-

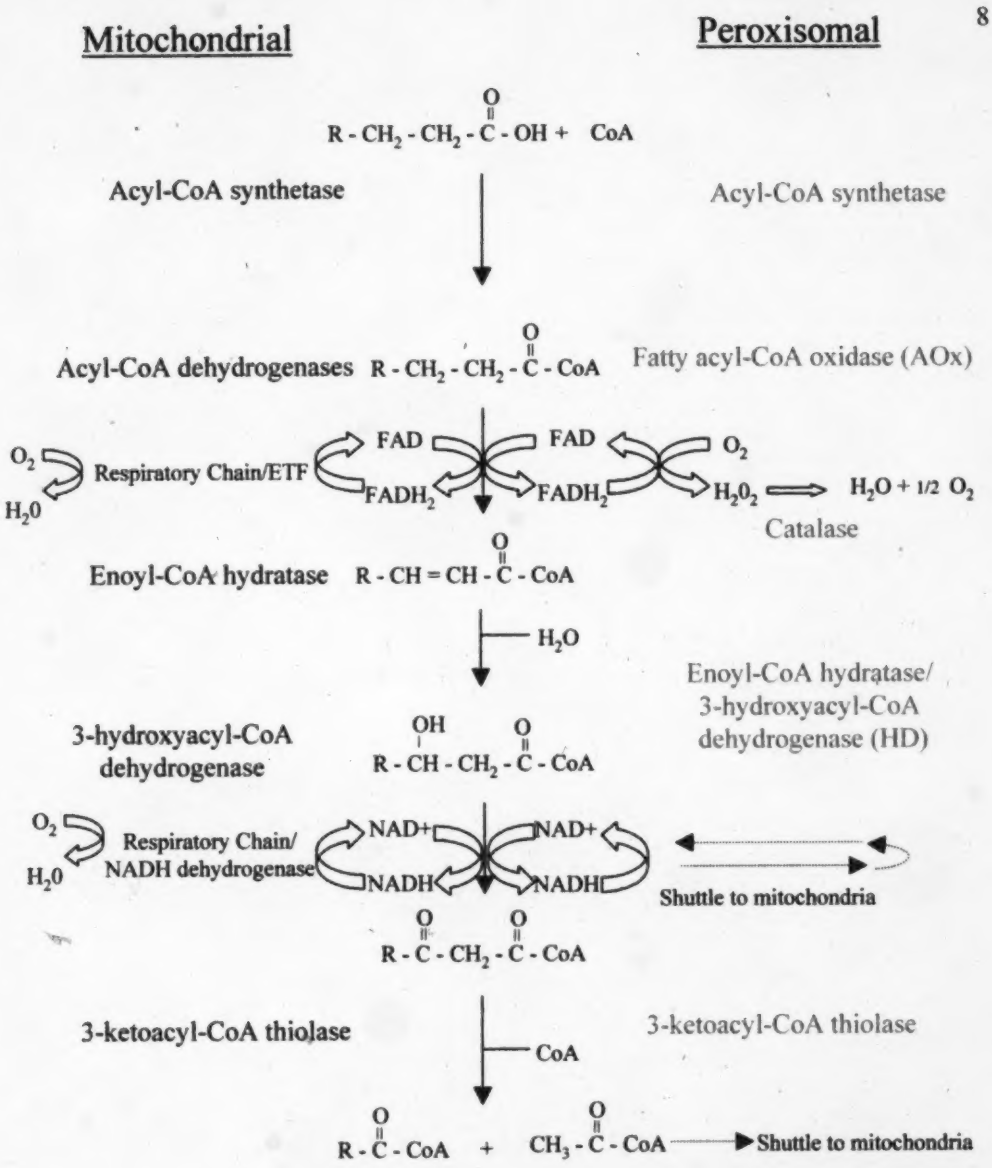


Figure 1-1 Comparison of peroxisomal and mitochondrial β-oxidation pathways.

oxidation, plants contain specialized peroxisomes referred to as glyoxysomes that are responsible for the β-oxidation of stored lipids and the conversion of triacylglycerols to glucose and essential metabolites (Hashimoto, 1996; Kindl, 1993; Lehninger et al., 1993). It is thought that the main function of mitochondrial β-oxidation in animals is the production of ATP, and that peroxisomal β-oxidation is important for the metabolism of toxic very long-chain fatty acids, medium chain dicarboxylic acids, cholesterol and unsaturated fatty acids that are not effectively oxidized in the mitochondria (Hashimoto, 1996; Mannaerts and van Veldhoven, 1996).

Administration of peroxisome proliferators increases the metabolic activity of peroxisomes and dramatically alters the peroxisomal enzyme content in a PPAR-dependent manner. In particular, the levels of the core enzymes of the peroxisomal β-oxidation cycle, AOx, HD and 3-ketoacyl-CoA thiolase, are significantly increased in response to peroxisome proliferators (Hijikata et al., 1990; McQuaid et al., 1987; Osumi, 1993; Ozasa et al., 1985; Reddy et al., 1986a; Thangada et al., 1989). Interestingly, the level of peroxisomal catalase is not concomitantly increased with the increases in the core β-oxidation enzymes. This dysregulation is thought to lead to an increase in highly reactive H_2O_2 radicals, and has been proposed to be responsible for the hepatocarcinogenic effects of peroxisome proliferators through oxidative DNA damage (Rao and Reddy, 1991; Reddy and Rao, 1989; Reddy et al., 1986b). Given that many fatty acids function as peroxisome proliferators, this provides a feedback loop to stimulate β-oxidation and ω-hydroxylation in response to elevated fatty acid levels, and to govern the inhibition of β-oxidation in conditions where fatty acid levels are reduced.

1.4 Nuclear Receptor Response Elements

Members of the nuclear receptor superfamily mediate their actions on transcription by binding to response elements located within the promoter regions of target genes (Evans, 1988; Gronemeyer, 1992; Keightley, 1998; Meier, 1997; Parker, 1993; Umesono and Evans, 1989). PPARs belong to the retinoid/vitamin D3/thyroid receptor subfamily of nuclear receptors that recognize core binding sites consisting of repeats of the consensus sequence TGACCT (Latruffe and Vamecq, 1997; Schoonjans et al., 1996). Several layers of specificity are contained within the arrangement of nuclear receptor response elements, and include different requirements in terms of the orientation, spacing, core consensus sequence and sequences flanking the TGACCT-like repeats (Castelein et al., 1997; IJpenberg et al., 1997; Juge-Aubry et al., 1997; Mader et al., 1993; Olson and Koenig, 1997; Osada et al., 1997; Umesono et al., 1991).

1.4.1 Peroxisome Proliferator Response Elements

PPAR heterodimerizes with 9-*cis*-retinoic acid receptor (RXRα) and binds preferentially to PPREs that contain 1 nucleotide spacing between direct tandem repeats, referred to as DR1 spacing (Chu et al., 1995a; Gearing et al., 1993; Keller et al., 1993; Kliewer et al., 1992b). It has been reported that PPAR/RXR heterodimers favour PPREs in which the separating nucleotide is A or T (Johnson et al., 1996; Juge-Aubry et al., 1997; Palmer et al., 1995). The absolute requirement for orientation and spacing varies widely between different nuclear receptors (Glass, 1994; Green, 1993; Mader et al., 1993). The thyroid hormone (TR) and retinoic acid receptors (RAR) bind to response elements which

typically contain DR3, 4 or 5 spacing. TR also recognizes sites consisting of palindromic or inverted palindromic repeats with variations in spacing (Desvergne, 1994; Lazar et al., 1991b; Ribeiro et al., 1995b; Tsai and O'Malley, 1994). Other nuclear receptors exhibit greater promiscuity in binding to response elements. The chicken ovalbumin upstream promoter transcription factor (COUP-TF) represses transactivation by binding to a large number of differentially spaced and oriented repeats (Cooney et al., 1993; Cooney et al., 1992; Miyata et al., 1993) This variation in binding preferences accounts for the interplay between different nuclear receptors through competition for shared target sites. Additionally some nuclear receptors can accommodate alterations in the core TGACCT-like motif and will bind readily to response elements that harbor variations in the core sequence (Green, 1993; Meier, 1997). PPREs have been characterized that contain minor variations in sequence, yielding a broad consensus site of TGAC/ACR (IJpenberg et al., 1997; Juge-Aubry et al., 1997; Palmer et al., 1995).

It is becoming more apparent that the sequences adjacent to the core sites within PPREs exhibit a role in directing the binding of PPAR/RXR heterodimers to these sites (Castelein et al., 1997; IJpenberg et al., 1997; Juge-Aubry et al., 1997; Osada et al., 1997, Palmer et al., 1995). Studies of a number of natural and synthetic PPREs that examined additional sequence requirements and heterodimer polarity determined that PPAR binds to the 3' TGACCT-like site and RXR binds to the 5' repeat (Chu et al., 1995a; IJpenberg et al., 1997; Juge-Aubry et al., 1997). This is the opposite binding arrangement of most other RXR heterodimerization partners such as TR and RAR, and may be due to intrinsic differences within specific response elements and to sites within the heterodimerization interfaces of

PPAR and RXR. Interestingly, the sequence flanking the 3' TGACCT-like repeat has been demonstrated to direct differential binding of distinct PPAR isoforms. PPARγ binds to, and transactivates readily from, the adipocyte lipid-binding protein AP2 response element (ARE6)-PPRE, whereas PPARα is unable to associate with this response element (Juge-Aubry et al., 1997). Replacement of only the ARE6-PPRE 3' flanking region with that of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) synthase-PPRE enables the binding of PPARα to this hybrid response element (Juge-Aubry et al., 1997). The arrangement of response elements permits interplay between nuclear receptors based on specificity at the level of DNA binding. Alterations in the core sequence, spacing and orientation of TGACCT-like repeats and flanking sequences, coupled with the degree of DNA binding promiscuity of certain nuclear receptors, enable the differential regulation of target genes by distinct nuclear receptors via their respective response elements (Glass, 1994; Naar et al., 1991).

1.4.2 Regulation of Genes Encoding peroxisomal β-Oxidation Enzymes

Peroxisome proliferators were shown to induce the levels of peroxisomal β-oxidation enzymes and subsequently found to increase transcription of a number of genes, including those encoding enzymes of the peroxisomal β-oxidation pathway (Dreyer et al., 1993; Lazarow and De Duve, 1976; McQuaid et al., 1987; Osumi and Hashimoto, 1978; Reddy et al., 1986a). Closer examination resulted in the identification of PPREs in the promoter/enhancer regions of the AOx and HD genes (Osumi et al., 1991; Tugwood et al., 1992; Zhang et al., 1993; Zhang et al., 1992). There are two DR1 sites upstream of the AOx gene (between -580 and -516 nucleotides upstream of the site of transcription

initiation)(Osumi et al., 1991), however, only the most distal DR1 was found to be bound by PPAR/RXR and to potentiate transactivation in response to peroxisome proliferators (Tugwood et al., 1992). The HD-PPRE contains four tandem TGACCT-related repeats located between nucleotides -2956 to -2919 upstream of the site of transcription initiation of the HD gene (Zhang et al., 1992). This region is sufficient to confer responsiveness to peroxisome proliferators and for binding of PPAR/RXR heterodimers (Zhang et al., 1993). The HD-PPRE is more complex than the AOx-PPRE, as it consists of two DR1 sites separated by 2 nucleotides, yielding an internal DR2 site (Chu et al., 1995a; Zhang et al., 1993; Zhang et al., 1992). This arrangement increases the potential for competitive interactions between nuclear receptors with DR1 and DR2 core site requirements. The 3' region flanking the second TGACCT repeat of the DR1 is important for both the binding of, and transactivation by, PPAR/RXR, with a broad consensus of TGACCTAG(A/T)A being deduced by comparing numerous PPREs (Johnson et al., 1996; Osada et al., 1996). The A/T at position +3 is the most conserved nucleotide among the PPREs and appears to be essential for efficient binding of, and transactivation by, PPAR/RXR heterodimers (Johnson et al., 1996; Osada et al., 1996).

1.5 PPARs and the Nuclear Receptor Superfamily

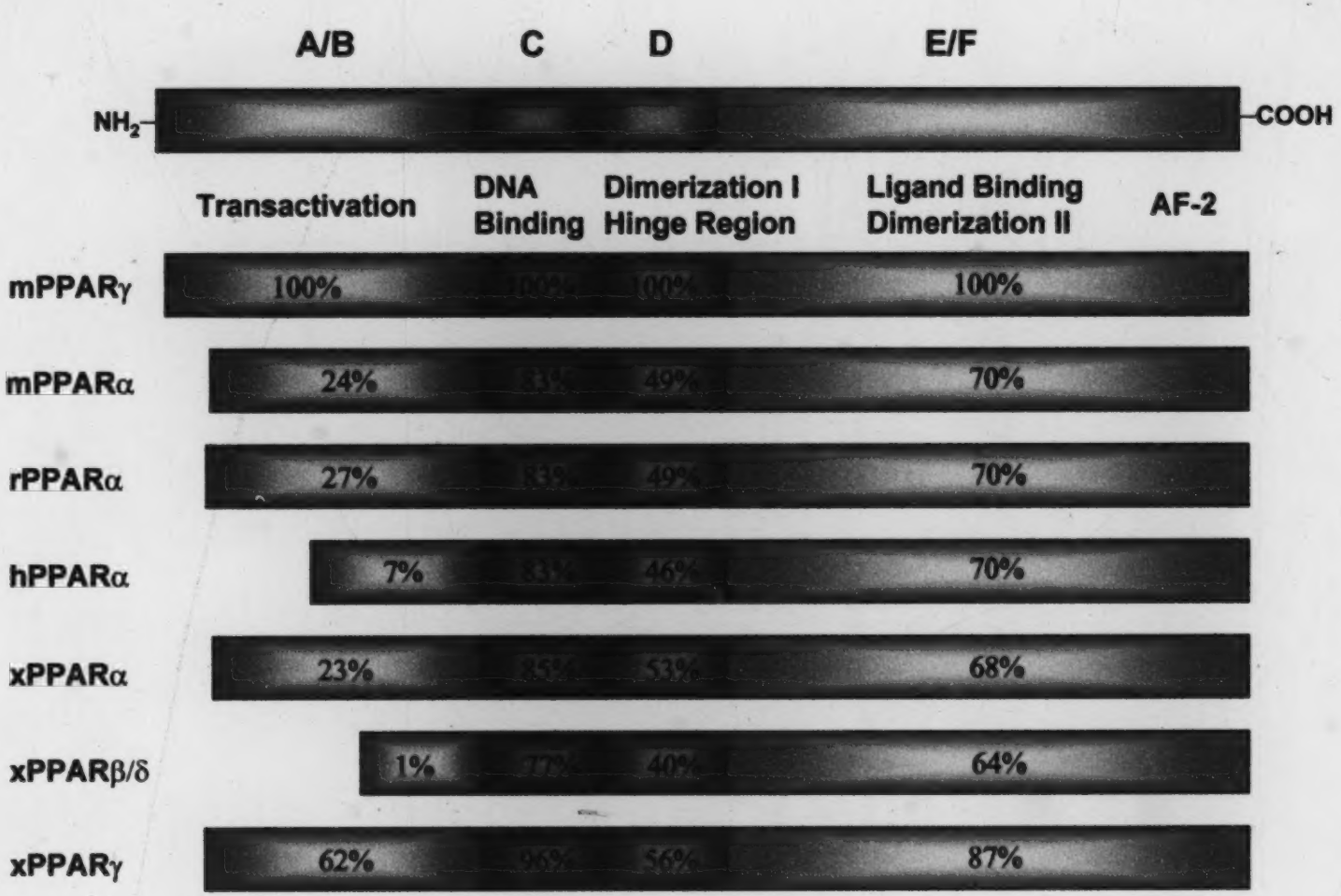
The nuclear receptor superfamily encompasses a large number of members, which are further classified into subfamilies based on their DNA-binding domains and their homology to other nuclear receptors (Keightley, 1998; Meier, 1997; Moras and Gronemeyer, 1998; Stunnenberg, 1993). The PPARs form a group of nuclear receptors within the vitamin

D3/thyroid hormone/retinoid receptor subfamily. PPARs have been identified in a number of species including human (Schmidt et al., 1992; Sher et al., 1993), rat (Gottlicher et al., 1992), mouse (Chen et al., 1993; Issemann and Green, 1990; Tontonoz et al., 1994b; Zhu et al., 1993), guinea pig (Bell et al., 1998), hamster (Aperlo et al., 1995), salmon (Ruyter et al., 1997) and *Xenopus laevis* (Dreyer et al., 1992). Three PPAR isoforms have been characterized: α, β/δ and γ, and each of these isoforms has a specific tissue distribution and regulates transcription of both distinct and overlapping target genes (Latruffe and Vamecq, 1997; Schoonjans et al., 1996).

1.5.1 General Structure of Nuclear Receptors

Nuclear receptors contain distinct domains that are functionally interchangeable (Glass, 1994; Laudet et al., 1992; Stunnenberg, 1993). The A/B domain is localized near the amino terminus, shows the most variation among nuclear receptors and is necessary for transactivation (see Fig. 1-2). The C region is highly conserved, contains two zinc-finger motifs required for DNA-binding and also harbors a dimerization interface. The P-box sequence is located at the carboxy-terminal end of the first zinc finger and confers DNA-binding specificity. The P-box is highly conserved and serves as a means to subclassify nuclear receptors based on similarities within this region. Members of the thyroid/retinoid/peroxisome proliferator receptor family all contain an identical P-box motif of CEGCKG. The D region is less conserved and is characterized by a hinge domain. The ligand binding domain is found in the E/F region near the carboxy terminus. Within the ligand binding domain lies the activation function-2 (AF-2) region, which has been shown to be

Figure 1-2. Nuclear receptor structure and comparison of PPAR isoforms. The modular structure of members of the nuclear receptor superfamily is illustrated. The location of several common domains are indicated and include the NH_2-terminal transactivation domain, DNA- and ligand-binding domains, both dimerization interfaces, the hinge region and the activation function-2 (AF-2) domain. PPAR isoforms from mouse (m), rat (r), human (h) and *Xenopus laevis* (x) are shown. The information contained in this figure is derived from papers by Forman et al., 1996, Dreyer et al., 1993, Forman et al., 1995b, Lazar, 1993 and Glass, 1994.


A/B
C
D
E/F
NH2
COOH
Transactivation
DNA Binding
Dimerization I Hinge Region
Ligand Binding Dimerization II
AF-2
mPPARγ
100%
100%
100%
mPPARα
24%
49%
70%
rPPARα
27%
49%
70%
hPPARα
7%
46%
70%
xPPARα
23%
53%
68%
xPPARβ/δ
1%
40%
64%
xPPARγ
62%
56%
87%

essential for interactions between nuclear receptors and nuclear receptor co-regulatory factors. A second nuclear receptor dimerization interface exists within the ligand binding domain. Structural studies of the PPARγ ligand binding domain revealed a large ligand binding cavity, and this may explain how a variety of structurally diverse ligands can activate this receptor (Moras and Gronemeyer, 1998; Nolte et al., 1998).

1.5.2 Dimerization Requirements

Nuclear receptors bind their response elements primarily as homo- or heterodimers; however, some receptors can bind as monomers (Carlberg et al., 1994; Keightley, 1998; Maruyama et al., 1998; Meier, 1997; Moras and Gronemeyer, 1998). PPARs require heterodimerization with the 9-*cis*-retinoic acid receptor (RXRα) to enable association with PPREs (Gearing et al., 1993; Kliewer et al., 1992a). Nuclear receptors have two putative dimerization interfaces, one within the ligand binding domain and a second interface within the DNA binding domain (Lee et al., 1998; Perlmann et al., 1996). The recently characterized 40 amino acid ligand binding domain dimerization interface contains an 11 amino acid core region that is sufficient for directing dimerization preferences in a yeast two hybrid system (Lee et al., 1998). A two step method for nuclear receptor activation has been postulated, in which interaction between ligand binding domain-dimerization interfaces allows formation of dimers in solution capable of binding a range of DNA elements (Perlmann et al., 1996). This initial interaction is followed by contact between the DNA binding domain-dimerization interfaces which restricts DNA-binding specificity (Perlmann et al., 1996).

1.5.3 Orphan Receptors and the Role of Ligand Binding

The largest group of nuclear receptors are those for which no ligand has been identified, or may not exist (Keightley, 1998; Meier, 1997). These are referred to as orphan receptors. Nuclear receptors are activated by ligand binding; however, ligands are not required for binding of nuclear receptors to target DNA response elements (Keightley, 1998; Meier, 1997; Tenbaum and Baniahmad, 1997). The search for nuclear receptor ligands is being performed by numerous groups, some relying on the observation that *in vitro* binding affinity of nuclear receptors towards co-regulatory factors, termed co-activators, greatly increases in the presence of ligand (Desvergne et al., 1998; Krey et al., 1997). The ligand binding requirement for association of the steroid receptor co-activator-1 (SRC-1) with PPAR has allowed the development of a co-activator-dependent receptor ligand assay (Desvergne et al., 1998; Krey et al., 1997; Willson and Wahli, 1997). This assay has identified several natural fatty acids and hypolipidemic drugs as ligands for *Xenopus* PPARα, β/δ, and γ. Additional assays based on conformational changes and using radiolabelled agonists have also been critical in determining PPAR ligands (Forman et al., 1997a; Kliewer et al., 1997). The identification of specific agonists and antagonists will prove fruitful in future experiments in order to determine the genes that are regulated by specific receptors and for characterization of novel co-regulatory interactions.

1.6 The Peroxisome Proliferator Activated Receptors

The PPAR family of nuclear receptors contains three isoforms, α, γ and δ, that each exhibit a distinct pattern of expression. These nuclear receptors have demonstrated roles in

the regulation of lipid metabolism and homeostasis, the control of differentiation, oncogenesis and the progression of apoptosis (Cherkaoui Malki et al., 1990; Chinetti et al., 1998; Latruffe and Vamecq, 1997; Peters et al., 1997a; Tontonoz et al., 1998).

1.6.1 PPARα

PPARα is found primarily in liver, kidney, heart and brain tissues, and has a critical role in the regulation of genes involved in lipid metabolism and homeostasis (Forman et al., 1996; Issemann and Green, 1990; Latruffe and Vamecq, 1997; Schoonjans et al., 1996). Among the genes regulated by PPARα are those responsible for peroxisomal β-oxidation: AOx (Osumi et al., 1991; Tugwood et al., 1992) and HD (Zhang et al., 1993; Zhang et al., 1992), ω-hydroxylation: cytochrome P450 (CYP) 4A1 and 4A6 (Aldridge et al., 1995; Muerhoff et al., 1992), and fatty acid transport: fatty acid binding protein (Poirier et al., 1997), acyl CoA synthetase (Schoonjans et al., 1995), long chain fatty acid translocase and fatty acid transporter protein (Motojima et al., 1998), as well as apolipoproteins AI and CIII (Peters et al., 1997b; Vu-Dac et al., 1998), carnitine palmitoyl transferase (Mascaro et al., 1998), and mitochondrial HMG-CoA synthase (Rodriguez et al., 1994). In addition, PPARα has been implicated in the regulation of Cu^{2+},Zn^{2+}superoxide dismutase (Inoue et al., 1998), nitric oxide synthase (Colville-Nash et al., 1998), non-peroxisomal transthyretin and α-2u-globulin (Motojima et al., 1997). Examination of transgenic PPARα-deficient mice demonstrated prolonged inflammatory responses and increased levels of total serum cholesterol and apolipoprotein AI compared to those found in wild type animals (Peters et al., 1997b). These findings strengthen the proposed roles of PPARα in lipid homeostasis and in

inflammation. PPARα has also been linked to apoptosis, since activation of PPARα by nafenopin prevents apoptosis in hepatocytes through inhibition of bcl-2 activity (Christensen et al., 1998). The suppression of apoptosis is thought to be a contributing factor in the tumourogenic effect of peroxisome proliferators. PPARα is activated by certain fatty acids, fibrate hypolipidemic drugs and eicosanoids, with the hypolipidemic drug Wy-14,643 and leukotriene B_4 being shown to bind directly to and activate the PPARα isoform (Forman et al., 1997a; Kliewer et al., 1997; Krey et al., 1997; Schoonjans et al., 1997).

1.6.2 PPARγ

PPARγ is most abundant in fat tissue and has been named a master regulator of adipogenesis (Fajas et al., 1998; Hu et al., 1995; Tontonoz et al., 1994b; Tontonoz et al., 1994c). PPARγ is transiently expressed in cells of the nervous system during development and may co-operate with PPARδ in neural differentiation (Braissant and Wahli, 1998; Cullingford et al., 1998; Granneman et al., 1998). Additionally, PPARγ and PPARα appear to be involved in the differentiation and apoptotic programs of certain cells of the immune system, with possible roles in atherogenesis and arthritis (Chinetti et al., 1998; Christensen et al., 1998; Elstner et al., 1998; Jiang et al., 1998; Ricote et al., 1998; Tontonoz et al., 1998). Target genes for PPARγ include adipocyte lipid-binding protein 2 (Tontonoz et al., 1994a; Tontonoz et al., 1994b), phosphoenolpyruvate carboxykinase (Tontonoz et al., 1995), uncoupling proteins 1 and 2 (Aubert et al., 1997; Camirand et al., 1998; Digby et al., 1998), and long chain fatty acid transporter protein (Martin et al., 1997). The importance of PPARγ in differentiation first became apparent following the demonstration of Spiegelman and

colleagues that overexpression of PPARγ and CCAAT/enhancer binding protein α was sufficient to induce the adipogenic program not only in preadipocytes, but also in normally non-adipogenic cells (Hu et al., 1995; Tontonoz et al., 1994c). A number of compounds that activate PPARγ have been discovered and include the thiazolidinediones class of antidiabetic compounds (Krey et al., 1997; Lehmann et al., 1995). It has further been shown that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and thiazolidinedione BRL49653 are high-affinity ligands that activate PPARγ through direct binding interactions (Forman et al., 1995a; Kliewer et al., 1995; Kliewer et al., 1997; Nolte et al., 1998). Particular attention has focussed on the effects of the thiazolidinediones, since they are insulin sensitizers used in the treatment of type II diabetes (Ibrahimi et al., 1994; Lehmann et al., 1995). Recently, a clinical investigation has linked mutations at Ser114 in human PPARγ with obesity, and it was further demonstrated that this mutation accelerates adipocyte differentiation *in vitro* (Ristow et al., 1998).

1.6.3 PPARδ

Although PPARδ has a very broad tissue distribution, little is known about the target genes and specific activators of this PPAR isoform. PPARδ is highly expressed in the nervous system, and it appears that it plays a role in oligodendrocyte and astrocyte differentiation (Cullingford et al., 1998; Granneman et al., 1998). PPARδ may co-operate with PPARγ in tissue-specific regulation of certain genes, including the uncoupling protein-2 gene (Aubert et al., 1997). PPARδ is weakly activated by a number of peroxisome proliferators including Wy-14,643 and the thiazolidinediones BRL49653; however, L-631,033 cinnamic acid has been identified as a potent PPARδ-specific activator (Johnson et al., 1997).

1.7 Hepatocyte Nuclear Factor-4

Hepatocyte nuclear factor-4 (HNF-4) is an orphan nuclear receptor that is present primarily in liver and kidney, and in lower amounts in brain, spleen and lung. This tissue distribution is similar to that observed for peroxisome proliferator-responsiveness (Dreyer et al., 1992; Issemann and Green, 1990). HNF-4 binds to DR1 response elements as a homodimer, and contains an extended consensus DNA binding site (Jiang et al., 1995; Sladek et al., 1990). The HNF-4 target DNA binding sequence is highly conserved within the AOx-PPRE and less conserved within the HD-PPRE (Osumi et al., 1991; Sladek et al., 1990; Tugwood et al., 1992; Zhang et al., 1992). Interestingly, it has been observed that fatty acyl-CoA thioesters with chain lengths greater than C12 act as ligands for HNF-4, further implicating HNF-4 in fatty acid regulation (Hertz et al., 1998).

Both PPAR and HNF families are key regulators of differentiation and metabolic pathways (Duncan et al., 1998; Latruffe and Vamecq, 1997; Schoonjans et al., 1996). A mutation in HNF-4 has been shown to be responsible for a form of early onset non-insulin dependent diabetes mellitus, indicating the importance of HNF-4 in glucose metabolism (Duncan et al., 1998; Stoffel and Duncan, 1997; Yamagata et al., 1996). Genes which are regulated by HNF-4 include the glycolytic enzymes aldolase B and glyceraldehyde-phosphate dehydrogenase, as well as liver pyruvate kinase and glucose transporter 2 (Gregori et al., 1998; Stoffel and Duncan, 1997). PPAR and HNF-4 co-regulate several genes responsible for fatty acid metabolism and homeostasis including apolipoprotein AI (Jiang et al., 1995; Peters et al., 1997b; Vu-Dac et al., 1998), fatty acid binding protein (Poirier et al., 1997; Stoffel and Duncan, 1997), mitochondrial HMG-CoA synthase (Rodriguez et al., 1994;

Rodriguez et al., 1998), liver transferrin (Hertz et al., 1996b) and apolipoprotein CIII (Fraser et al., 1998; Hertz et al., 1995). These nuclear receptors also affect the transcription of cytochrome P450 (CYP) genes, with HNF-4 modulating expression of CYP 2A4 (Yokomori et al., 1997) and CYP 3A23 (Huss and Kasper, 1998) and PPAR shown to influence transcription of CYP 4A1 and 4A6 (Aldridge et al., 1995; Johnson et al., 1996; Muerhoff et al., 1992). A list of processes which are governed by both HNFs and PPARs is rapidly emerging. In addition to directing the expression of genes involved in lipid homeostasis, these two receptor families have been shown to control genes encoding enzymes responsible for inflammation (Chinetti et al., 1998; Jiang et al., 1998; Peters et al., 1997b; Ricote et al., 1998; Ueda et al., 1998), and overlap in the control of certain viral genes including the pre-C and nucleocapsid promoters of hepatitis B virus (Raney et al., 1997; Yu and Mertz, 1997) and the composite element in the long terminal repeat of HIV-I isolates (Ladias, 1994).

1.8 Thyroid Hormone Receptors

The thyroid hormone receptor (TR) family is a well characterized group of nuclear receptors which are activated 3, 3', 5-triiodothyronine (T3)(Evans, 1988; Lazar, 1991a; Ribeiro et al., 1995a; Shepard and Eberhardt, 1993) The actions of TR are widespread and this nuclear receptor family has been shown to be at the centre of regulating many cellular processes including lipid metabolism, cell growth and differentiation (Chatterjee and Tata, 1992; Desvergne, 1994; Goodridge, 1987; Silva and Rabelo, 1997; Xiong et al., 1998). The wide ranging effects of TR family members are due in part to the broad response element preference exhibited by these receptors. Thyroid hormone response elements may contain

direct, palindromic and inverted palindromic repeats with a range of spacing (Desvergne, 1994; Ribeiro et al., 1998; Ribeiro et al., 1995b; Tsai and O'Malley, 1994); however, the nucleotides flanking the TGACCT-related sites appear to influence TR binding (Olson and Koenig, 1997). In addition, TRs can function as monomers, homodimers and as heterodimers with RXRα (Desvergne, 1994; Ikeda et al., 1994; Lazar et al., 1991b; Tsai and O'Malley, 1994).

The TR family has been the focus of much of the pioneering work which has established general mechanisms of nuclear receptor function. In particular, the ability of TRs to function as transcriptional silencers has driven much of the early research and discovery of nuclear receptor accessory factors, and is discussed in more detail in a subsequent section (Baniahmad et al., 1992; Baniahmad et al., 1995; Damm et al., 1989; Graupner et al., 1989; Renkawitz, 1993; Sap et al., 1989). A member of the TR family was a natural choice as a candidate regulator for peroxisome proliferator responsive genes, based on overlapping tissue distributions and the observations that transcription of several thyroid hormone-responsive genes were shown to be modulated by PPARα (Bogazzi et al., 1994; Castelein et al., 1994; Jow and Mukherjee, 1995). Genes which are co-regulated by PPARα and TRα include those encoding malic enzyme, CYP 4A family proteins and uncoupling proteins (Aldridge et al., 1995; Dogra et al., 1998; IJpenberg et al., 1997; Johnson et al., 1996; Muerhoff et al., 1992; Silva and Rabelo, 1997; Webb et al., 1996).

1.9 Retinoid-like Z Receptor/Retinoid Orphan Receptors

The retinoid-like Z receptor/retinoid orphan receptor (RZR/ROR) family consists of

three isoforms: α, β, and γ, which demonstrate broad, brain and skeletal muscle tissue distributions, respectively. The α isoform includes four splice variants (RORα1, RORα2, RORα3 and RZRα) (Becker-Andre et al., 1993; Carlberg et al., 1994; Giguere et al., 1994; Hirose et al., 1994). It has been reported that RZRs are activated directly by melatonin, however this finding has been subject to discussion (Becker-Andre et al., 1994; Carlberg and Wiesenberg, 1995; Hazlerigg et al., 1996; Rafii-El-Idrissi et al., 1998; Steinhilber et al., 1995). Recently, it has also been reported that several thiazolidinedione-like agents function as RZR ligands (Missbach et al., 1996; Wiesenberg et al., 1998). Members of this family of nuclear receptors bind as monomers to target response elements and have been shown to regulate genes involved in inflammation, differentiation and lipid metabolism (Carlberg et al., 1994; Giguere et al., 1994; Hirose et al., 1994; Schrader et al., 1996; Vu-Dac et al., 1997). The α and γ isoforms are induced early in adipogenesis suggesting involvement in pre-adipocyte differentiation and possible interplay with the adipogenic regulator, PPARγ (Austin et al., 1998; Spiegelman and Flier, 1996). Monomeric receptors from the NGFI-B and RZR/ROR families contain a carboxy-terminal extension of the zinc finger in their DNA binding domains (Wilson et al., 1992). This extended region is also found in the DNA-binding domain of PPAR, and is required for the recognition of extended 3' sequences that flank TGACCT-like core sites (Hsu et al., 1998; Wilson et al., 1992). The RZR consensus flanking sequence is similar to those which border a number of nuclear receptor response elements including several PPREs (Schrader et al., 1996). The HD-PPRE was identified as a possible RZR regulatory site in a large scale screen for candidate RZR response elements (Schrader et al., 1996).

1.10 Calreticulin

Calreticulin is a Ca^{2+}-binding protein which exhibits strong endoplasmic reticulum localization and is involved in a variety of cellular processes (Coppolino and Dedhar, 1998; Krause and Michalak, 1997). Calreticulin shares homology with the endoplasmic reticulum chaperone protein calnexin, and has demonstrated an important role as a chaperone involved in the proper folding and assembly of proteins within the endoplasmic reticulum (Vassilakos et al., 1998). Calreticulin can replace calnexin in the processing of mouse class I histocompatiblity molecules (Krause and Michalak, 1997; Vassilakos et al., 1998). Calreticulin readily binds Ca^{2+} and is thought to serve as a key factor in the storage and regulation of intracellular Ca^{2+} (Mery et al., 1996). Ca^{2+} is an extremely important cell signalling molecule and as a consequence calreticulin may be a central player governing signalling events, both within the endoplasmic reticulum lumen and at other locations within the cell. It has been demonstrated that Ca^{2+}-binding by calreticulin leads to direct interaction with, and sequestration of, another endoplasmic reticulum lumenal protein, protein disulfide isomerase, suggesting a regulatory role for calreticulin via Ca^{2+}-dependent protein-protein interactions (Baksh et al., 1995). Calreticulin has also been implicated in the regulation of cell adhesion to extracellular substrata (Leung-Hagesteijn et al., 1994; Opas et al., 1996). Although calreticulin can interact *in vitro* with KLGFFKR motifs in the cytoplasmic tail of α-integrin, it appears that calreticulin is affecting adhesion indirectly by altering the level of vinculin (Opas et al., 1996; Rojiani et al., 1991). A role for calreticulin in regulation of gene expression has also been suggested since, *in vitro*, calreticulin binds to KXXFF(K/R)R motifs localized in the DNA-binding domains of nuclear receptors. Calreticulin interacts with these

motifs in the DNA-binding domains of glucocorticoid, vitamin D3, retinoic acid and androgen receptors *in vitro*, and inhibits transactivation by these receptors in transfection experiments (Burns et al., 1994; Dedhar et al., 1994; Desai et al., 1996; Michalak et al., 1996; St-Arnaud et al., 1995; Wheeler et al., 1995). Consequently it was proposed that calreticulin is a general regulator of nuclear receptor activity through physical interactions with the conserved KXXFF(K/R)R motif located in the DNA binding domains of nuclear receptors (Burns et al., 1994; Dedhar et al., 1994; Shago et al., 1997).

1.11 Nuclear Receptor Co-Regulatory Factors

In the past three years the intense interest in nuclear receptors and the molecular means by which they influence transcription has resulted in the discovery of a novel class of nuclear receptor accessory factors (Horwitz et al., 1996; Nolte et al., 1998; Torchia et al., 1998). These co-regulatory proteins serve to direct the potentiation or inhibition of transcription through physical interactions with nuclear receptors, and are referred to as co-activators and co-repressors, respectively. By biochemical and structural examinations, a common mechanism of nuclear receptor regulation is emerging (Chen and Li, 1998; Lavinsky et al., 1998; Nolte et al., 1998; Westin et al., 1998). It appears that ligand binding causes a structural rearrangement in the nuclear receptor which results in the dissociation of a co-repressor complex and facilitates direct interaction between ligand-bound receptors and a co-activator complex. In addition to interacting with nuclear receptors, co-repressors and co-activators are directly involved in chromatin remodelling.

1.11.1 The Activation Function Region

Nuclear receptors contain a discrete ligand binding domain within their carboxy-terminus. Through mutational studies with the estrogen receptor (ER), a region within the ligand binding domain was found to be absolutely necessary for transactivation (Danielian et al., 1992). This region was named the activation function-2 (AF-2) domain. Insights into structural alterations within the ligand binding domain following ligand association were obtained by performing X-ray crystallography of a number of nuclear receptors in the unbound, agonist- or antagonist-bound state (Bourguet et al., 1995; Brzozowski et al., 1997; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995). A high degree of structural conservation in the ligand binding domains of the nuclear receptors was observed. These regions consist of 12 alpha helices with a conserved beta-turn between helices 5 and 6 (Moras and Gronemeyer, 1998; Nolte et al., 1998; Torchia et al., 1998). Interestingly, the AF-2-containing helix 12 undergoes dramatic alterations in response to agonist or antagonist binding. In comparisons of the ligand binding domains of RAR, RXR, ER, PPARγ and TR, agonist binding appears to cause helix 12 to adopt a conformation in which it directly interacts with the ligand, suggesting that AF-2 switches between different conformations in the presence or absence of ligand (Bourguet et al., 1995; Brzozowski et al., 1997; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995). Binding of the ER antagonist, raloxifene, causes helix 12 to become partially buried and obscures residues known to be necessary for transcriptional activation (Brzozowski et al., 1997). PPARα undergoes a conformational change following exposure to agonists, based on protease sensitivity assays (Dowell et al., 1997b). Structural data from studies with PPARγ and its ligand BRL49653 demonstrate the

importance of helix 12 structural rearrangements to facilitate interactions with the SRC-1 and transcriptional intermediary factor 2 (TIF2) co-activators (Leers et al., 1998; Nolte et al., 1998; Westin et al., 1998). Other aspects of nuclear receptor ligand binding domains are also modified following association with ligand, suggesting that agonists and antagonists directly alter the surface structure of nuclear receptor ligand binding domains (Bourguet et al., 1995; Moras and Gronemeyer, 1998; Renaud et al., 1995; Torchia et al., 1998; Wagner et al., 1995).

1.11.2 Nuclear Receptor Co-Activators

A number of co-activator proteins have been identified which mediate transcriptional activation in a ligand- and AF-2-dependent manner. Co-activators were primarily isolated by screens for factors that interacted with ligand binding domains, or with epitope-tagged or ligand-bound nuclear receptors (Glass et al., 1997; Hong et al., 1996; Monden et al., 1997; Zhu et al., 1997). These studies showed the involvement of accessory factors in transcriptional activation by nuclear receptors. A number of related nuclear receptor co-activators have been isolated and include glucocorticoid receptor-interacting protein 1 (GRIP1) (Hong et al., 1996), activator of the thyroid and retinoic acid receptor (ACTR) (Chen et al., 1997), receptor-associated co-activator 3 (RAC3) (Li et al., 1997a), p300/CBP co-integrator associate protein (p/CIP) (Torchia et al., 1997), and TR-activator molecule (TRAM) (Takeshita et al., 1997). The co-activators SRC-1 (DiRenzo et al., 1997), receptor-interacting protein 140 (RIP140) (Treuter et al., 1998), PPARγ-binding protein (PBP) (Zhu et al., 1997), TIF2 (Leers et al., 1998) and PPARγ-co-activator (PGC) (Puigserver et al.,

1998) have been observed to interact with PPARs. CREB binding protein (CBP) and the functionally related p300 exhibit a more general co-activator function for both nuclear receptors and other transcription factors such as cAMP response element-binding protein (CREB), activator protein-1, and signal transducer and activator of transcription (STAT) (Arany et al., 1994; Chakravarti et al., 1996; Horvai et al., 1997; Kamei et al., 1996). CBP/p300 not only interacts with transcription factors, but also possesses intrinsic histone acetyl transferase activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). Chromatin remodelling through the acetylation of histones is thought to make DNA elements more accessible to transcriptional machinery and thereby increase transcription (Glass et al., 1997; Grunstein, 1997; Torchia et al., 1998; Wade et al., 1997; Wolffe, 1997; Wolffe, 1998). CBP/p300 interacts strongly with nuclear receptors, including PPARγ, in a ligand-dependent manner (Dowell et al., 1997a). The necessity of cooperation between nuclear receptors and CBP/p300 is evident since the majority of identified co-activators contain both nuclear receptor and CBP/p300-interaction domains (Ding et al., 1998; Schmidt et al., 1998; Takeshita et al., 1998; Voegel et al., 1998; Yao et al., 1996). Another factor which contains histone acetyl transferase activity, p300/CBP-associated factor (p/CAF), interacts with CBP/p300 and is a component of the proposed co-activator complex (Bannister and Kouzarides, 1996; Chen et al., 1997; Yang et al., 1996). It was recently observed that SRC-1 also functions as a histone acetyl transferase (Spencer et al., 1997). A common LXXLL motif (where L denotes leucine and X represents any amino acid) is present in co-activators and has been shown to be necessary for nuclear receptor/co-activator interactions (Ding et al., 1998; Heery et al., 1997; McInerney et al., 1998; Torchia et al., 1997; Voegel et al., 1998). It

appears that the LXXLL motifs are important for stabilizing interactions between ligand bound nuclear receptors and co-activators (Moras and Gronemeyer, 1998; Torchia et al., 1998; Westin et al., 1998). Addition of 25 amino acid fragments containing the LXXLL motif are sufficient to eliminate interactions between ligand-bound nuclear receptors and co-activators *in vitro,* and mutations in this motif eliminate co-activator mediated transcriptional activation by nuclear receptors in functional assays (Heery et al., 1997; Torchia et al., 1997; Westin et al., 1998). The co-activator complex is thought to consist of ligand bound nuclear receptors, CBP/p300, p/CAF, co-activators and as yet unidentified factors (Chen et al., 1997; Chen and Li, 1998; Glass et al., 1997; Westin et al., 1998). The association of ligand bound nuclear receptors with DNA results in structural changes that are thought to specify interactions with particular co-activators (Glass et al., 1997; Leers et al., 1998; Nolte et al., 1998; Torchia et al., 1998; Westin et al., 1998). Co-activator complexes may serve to increase transcription through several means including chromatin remodelling via histone acetylation, stabilization of nuclear receptor binding and facilitation of interactions with basal transcriptional machinery (Chen and Li, 1998; Glass et al., 1997; Horwitz et al., 1996; Torchia et al., 1998).

1.11.3 Nuclear Receptor Co-Repressors

RAR and TR act as transcriptional silencers for many genes in the absence of ligand (Baniahmad et al., 1992; Burcin et al., 1994; Damm et al., 1989; Graupner et al., 1989; Nawaz et al., 1995; Renkawitz, 1993; Sap et al., 1989). Closer examination of this observation resulted in the discovery of silencing units within the RAR and TR ligand binding

domains, and of co-repressors which mediate the silencing effects of TR and RAR (Chen and Evans, 1995; Kurokawa et al., 1995; Sande and Privalsky, 1996). The silencing activity of TR and RAR has been localized to a site within the ligand binding domain, but distinct from the AF-2 region, termed the CoR box (Baniahmad et al., 1992; Baniahmad et al., 1995; Zamir et al., 1996). Co-repressors inhibit transcription of target genes by several nuclear receptors in the unbound or antagonist-bound states (Chen and Evans, 1995; Jackson et al., 1997; Lee et al., 1995; Shibata et al., 1997; Smith et al., 1997). The nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) interact with a range of nuclear receptors (Chen and Evans, 1995; Heinzel et al., 1997; Lavinsky et al., 1998; Lee et al., 1995; Zamir et al., 1996; Zamir et al., 1997). Although NCoR and SMRT share homology, they show different preferences for specific nuclear receptors (Lavinsky et al., 1998). SMRT has been shown to repress transactivation by PPARs, whereas NCoR does not appear to interact with these receptors (Lavinsky et al., 1998; Yan et al., 1998; Zamir et al., 1997). Like co-activators, co-repressors also form complexes and these contain unliganded nuclear receptors, the co-repressor and factors responsible for chromatin remodelling (Heinzel et al., 1997; Laherty et al., 1997; Li et al., 1997b; Nagy et al., 1997; Torchia et al., 1998; Zhang et al., 1997). Recent investigation of core co-repressor complexes demonstrated that histone deacetylases and other components including Sin3 were present in the complex, and histone deacetylase activity was found to be required for re-establishment of the repressed chromatin state (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Laherty et al., 1998; Laherty et al., 1997; Nagy et al., 1997; Torchia et al., 1998; Zhang et al., 1997).

1.11.4 Model of Co-Activator/Co-Repressor Interactions

The current model of co-activator/co-repressor function proposes that DNA-bound nuclear receptors associate with a co-repressor complex, yielding histone deacetylation and transcriptional repression (see Fig. 1-3) (Chen and Li, 1998; Torchia et al., 1998). Upon ligand binding, the co-repressor complex is dissociated, allowing interaction of the ligand-bound nuclear receptor with a co-activator complex. This association enables histone acetylation and chromatin remodelling, revealing crucial DNA sites for transcriptional activation. In addition to being regulated by the availability and active state of nuclear receptors, it appears that additional signalling networks such as the mitogen-actvated protein kinase and protein kinase A cascades, may affect the function of co-activator and co-repressor complexes (Lavinsky et al., 1998; Torchia et al., 1998).

1.12 Nuclear Receptor Interplay

Interactions between nuclear receptors allow the integration of signals from diverse signalling pathways to direct transcriptional regulation of target genes. In experiments that directly compared the DNA element requirements of several nuclear receptors that recognize DR1 sites (including HNF-4, PPARα and RXRα), it was observed that the important factors influencing DNA binding affinity are determinants within the core sequence, the identity of the spacing nucleotide and the sequence flanking the core TGACCT-related repeats (Jiang et al., 1995; Juge-Aubry et al., 1997; Nakshatri and Bhat-Nakshatri, 1998; Nishiyama et al., 1998). It was also observed that DNA binding affinity correlates with transactivation potential from a given element (Nakshatri and Bhat-Nakshatri, 1998).

Figure 1-3. Model of nuclear receptor accessory factor interactions. In the absence of ligand or presence of antagonist, a co-repressor/nuclear receptor complex forms and maintains chromatin in the deacetylated (repressed) state. Binding of ligand (L) by the nuclear receptor results in a conformational change which enables dissociation of the co-repressor complex and assembly of a co-activator/nuclear receptor complex. The co-acativator complex contains histone acetyl-transferase activity which relieves chromatin repression and makes sites required for transcriptional initiation available, yielding transcriptional activation.

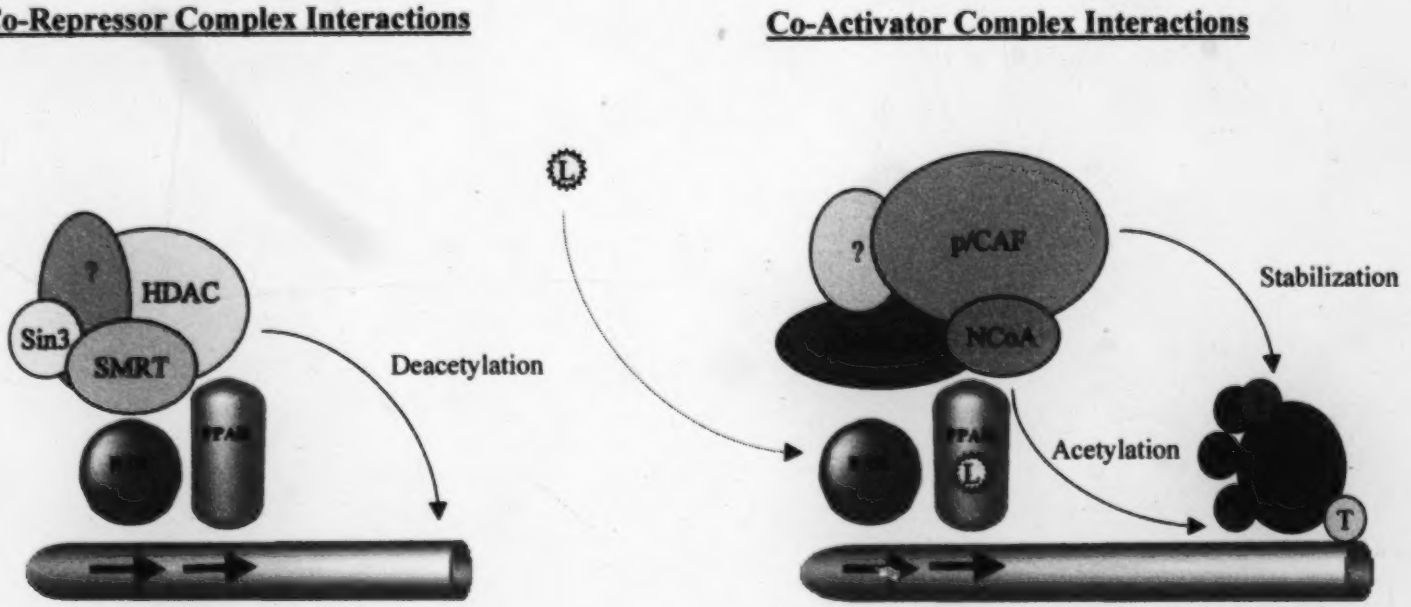


Figure 1-3. Model of nuclear receptor co-regulatory factor interactions.

The coordinate transactivation of target genes by nuclear receptors is governed by several levels of specificity in addition to those inherent in the DNA binding/response element requirements. Nuclear receptor interplay can be dictated by dimerization requirements, ligand binding, and by competition for common binding sites and for co-activators or co-repressors. Nuclear receptors typically bind to target response elements as homo- or heterodimers, however some receptors such as RZR, NGFB-I and TR can bind to DNA as monomers (Carlberg et al., 1994; Desvergne, 1994; Giguere et al., 1994; Ikeda et al., 1994; Lazar et al., 1991b; Maruyama et al., 1998). RXRα is a common heterodimerization partner for many nuclear receptors including PPAR (Kliewer et al., 1992b), RAR, TR (Zhang et al., 1992), and vitamin D3 receptor (VDR) (Thompson et al., 1998), and therefore the availability of RXRα can serve to alter the transactivation capacity of specific nuclear receptors due to competition for this heterodimerization partner (Chu et al., 1995b; DiRenzo et al., 1997; Juge-Aubry et al., 1995; Miyata et al., 1996).

The classical model of nuclear receptor activation describes ligand binding as a requirement for receptor activation, however ligand binding is not necessary for binding of nuclear receptors to their target response elements (Carson-Jurica et al., 1990; Evans, 1988). Ligand binding by nuclear receptors results in a conformational change in the AF2 domain which facilitates interaction with co-activators and is thought to displace associated co-repressors (Bourguet et al., 1995; Brzozowski et al., 1997; Moras and Gronemeyer, 1998; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995). It has been reported that ligand association also increases DNA binding of PPARα/RXRα heterodimers (Forman et al., 1997a). In the absence of ligand some nuclear receptors, such as TR, can repress

transcription. Therefore the availability of ligand critically affects transcriptional activation from target response elements.

Several different nuclear receptors can compete for binding to the same response element depending on their inherent promiscuity. COUP-TF is an orphan receptor that recognizes a wide variety of response elements and is a constitutive repressor of transcription. COUP-TF has been shown to compete for binding to response elements for PPAR, TR, RXR, RAR and VDR, and represses transactivation in part, through competition for shared response elements (Cooney et al., 1993; Cooney et al., 1992; Marcus et al., 1996; Miyata et al., 1993).

In recent years the discovery of accessory factors termed co-activators and co-repressors has added to our understanding of transcriptional regulation by nuclear receptors (Chen and Li, 1998; Glass et al., 1997; Horwitz et al., 1996; Nolte et al., 1998; Torchia et al., 1998; Westin et al., 1998). Nuclear receptors are dependent on interactions with accessory factors to potentiate or repress transactivation of target genes. In terms of nuclear receptor interplay, competition between nuclear receptors for accessory factors can affect the activity of nuclear receptors and thereby direct the activation or repression of a target response element.

1.13 Aims of This Project

In order to better understand the mechanisms involved in the regulation of the AOx and HD genes in response to peroxisome proliferators, we have examined the interplay between several key nuclear receptors in modulating gene expression. These studies have

investigated the interactions of PPARα, RXRα, HNF-4, TRα and RZRα in terms of recognition and binding of the AOx- and HD-PPREs and have been extended to examine the influences that these receptors exert in the transactivation of PPREs in a cellular context. I have also analyzed the role of the Ca^{2+}-binding protein, calreticulin, in the modulation of peroxisome proliferator-directed gene expression. The results of my investigation have enabled the formation of a more complete model of peroxisome proliferator-directed gene expression and have provided additional insights into the general mechanisms of nuclear receptor signalling.

CHAPTER 2

Transactivation of the Peroxisome Proliferator-Activated Receptor is Differentially Modulated by Hepatocyte Nuclear Factor-4[†]

[†]A version of this chapter has been published. Winrow, C.J., Marcus S.L., Miyata, K.S., Zhang, B., Capone, J.P. and Rachubinski, R.A. 1994. Gene Express. 4:53-62. Used with permission from Cognizant Communications Corporation.

2.1 ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) stimulate the expression of several genes involved in lipid metabolism by binding to specific *cis*-acting peroxisome proliferator-responsive elements (PPREs) via cooperativity with retinoid X receptors. We demonstrate here that hepatocyte nuclear factor-4 (HNF-4), another member of the nuclear hormone receptor superfamily, bound with differing affinities to the PPREs from the genes encoding rat acyl-CoA oxidase and hydratase-dehydrogenase, the first two enzymes of the peroxisomal ß-oxidation pathway. In cotransfection assays, HNF-4 repressed rat PPAR-dependent activation of a reporter gene linked to the acyl-CoA oxidase PPRE, either in the absence or presence of the peroxisome proliferator, Wy-14,643. Rat PPAR-dependent activation of a reporter gene linked to the hydratase-dehydrogenase PPRE was less efficiently repressed by HNF-4 in the absence of Wy-14,643 than was activation from the acyl-CoA oxidase PPRE. However, in the presence of Wy-14,643, HNF-4 functioned cooperatively with PPAR to significantly enhance induction from the hydratase-dehydrogenase PPRE. These results suggest that the genes encoding the first two enzymes of the peroxisomal ß-oxidation pathway are subject to differential regulation by the interplay of multiple members of the steroid/nuclear hormone receptor superfamily, mitigated in part by the structures of the PPREs and by the presence of activators of PPARs.

2.2 INTRODUCTION

Peroxisome proliferators constitute a large group of xenobiotic chemicals that include the fibrate family of hypolipidemic drugs, herbicides and phthalate ester plasticizers. Exposure to peroxisome proliferators increases both the number and metabolic capacity of peroxisomes (Lock et al., 1989). The mechanism of action of peroxisome proliferators is of considerable interest, since these agents have been shown to induce hepatomegaly, chromosomal aberrations and ultimately hepatocarcinogenesis in rodents (Rao and Reddy, 1991; Reddy, 1990; Reddy et al., 1980). Peroxisome proliferators do not mutate DNA directly and are therefore classified as non-genotoxic carcinogens.

The pleiotropic cellular responses to peroxisome proliferators are mediated in part by the transcriptional induction of a number of genes whose products are involved in lipid metabolism. These include the rat acyl-CoA oxidase (AOx) and hydratase-dehydrogenase (HD) genes, which encode the first two enzymes of the peroxisomal fatty acid ß-oxidation system (Reddy et al., 1986a); the CYP4A6 gene, which encodes a member of the cytochrome P450 fatty acid ω-hydroxylase family (Muerhoff et al., 1992); and the gene encoding the liver fatty acid-binding protein (Besnard et al., 1993; Issemann et al., 1992; Kaikaus et al., 1993). Transactivation of these peroxisome proliferator-responsive genes is mediated by peroxisome proliferator-activated receptors (Issemann and Green, 1990). PPARs are members of the steroid/thyroid hormone receptor superfamily that bind to specific *cis*-acting peroxisome proliferator-responsive elements (PPREs), resulting in an overall stimulation of transcription of responsive genes (Marcus et al., 1993; Osumi et al., 1991; Tugwood et al., 1992; Zhang et al., 1992). Human (Schmidt et al., 1992), rat (Gottlicher et al., 1992), mouse (Chen et al.,

1993; Issemann and Green, 1990; Zhu et al., 1993), and *Xenopus* (Dreyer et al., 1992) have been shown to harbor multiple PPAR-related genes, perhaps reflecting a requirement for different PPARs in regulating specific target genes or in mediating responsiveness to a variety of stimuli. PPARs can be activated by a wide spectrum of structurally diverse peroxisome proliferators, as well as by both synthetic and natural fatty acids (Dreyer et al., 1992; Keller et al., 1993). However, none of these agents binds directly to PPARs, and therefore the true ligands for these receptors remain to be identified.

The natural PPREs characterized so far consist of direct repeats of the core half-site motif TGACCT, which is also found in the cognate response elements of other nuclear hormone receptors of this class including the thyroid, retinoic acid, vitamin D3 and 9-*cis*-retinoic acid (RXR) receptors (Umesono et al., 1991). Target specificity is determined in part by the sequence of the half-sites, the number and relative spacing of their direct repeats, and the ability of some receptors to bind to cognate response elements as homodimers and/or heterodimers (Lucas and Granner, 1992; Mader et al., 1993). However, it is not uncommon for different nuclear hormone receptors to compete for binding to a particular DNA-response element. The potential for promiscuous binding, combinatorial interactions, and cross-talk among receptors serves to modulate transcription of hormone-responsive genes in multiple ways, thereby contributing to both the complexity and the diversity in signalling pathways. Indeed, we and others have demonstrated that PPARs bind to PPREs as heterodimers with 9-*cis*-retinoic acid receptors, implying that the peroxisome proliferator and retinoid signalling pathways converge (Gearing et al., 1993; Kliewer et al., 1992b; Marcus et al., 1993). Recently, we have shown that COUP-TF, an orphan member of the nuclear hormone receptor

superfamily, binds to the HD-PPRE and can antagonize rat (r) PPAR-mediated transactivation from this PPRE *in vivo* (Miyata et al., 1993). Therefore, PPAR function is dependent upon interactions with, and can be subject to modulation by, other members of the nuclear hormone receptor superfamily.

Hepatocyte nuclear factor-4 (HNF-4) is another orphan member of the nuclear hormone receptor family that binds to TGACCT-related elements (Sladek et al., 1990). HNF-4 is a liver-enriched factor that plays an important role in the transcriptional regulation of several genes whose products are involved in diverse metabolic pathways. These include the genes encoding apoAI, apoAII, apoB and apoCIII (Ladias et al., 1992; Mietus-Snyder et al., 1992), α_1-antitrypsin and transthyretin (Costa et al., 1989; Costa et al., 1988), ornithine transcarbamylase (Nishiyori et al., 1994), and some members of the cytochrome P450 hydroxylase superfamily (Chen et al., 1994). HNF-4 mRNA is most abundant in liver and kidney and low in brain, spleen, and lung, a pattern of expression closely matching peroxisome proliferator tissue specificities and PPAR abundance (Dreyer et al., 1992; Issemann and Green, 1990). Since peroxisomes are responsible for the metabolism of long-chain fatty acids, and the PPREs of both the AOx and HD genes share significant homology with the consensus HNF-4 target site, we explored the possibility that HNF-4 might play a role in the regulation of these genes. We demonstrate here that HNF-4 present in extracts of peroxisome proliferator-responsive rat hepatoma cells or synthesized *in vitro* bound strongly to the AOx-PPRE and with lower affinity to the HD-PPRE. HNF-4 on its own had no effect on the transcription of reporter genes linked to the AOx- or HD-PPRE; however, it repressed both the peroxisome proliferator-independent and peroxisome proliferator-dependent transactivation from the AOx-PPRE by PPARα. Surprisingly, while PPARα-dependent

transactivation of a HD-PPRE-linked reporter gene was also repressed by HNF-4, its activity was potentiated in the presence of the peroxisome proliferator Wy-14,643. Our results suggest that HNF-4 plays an important role in the regulation of genes encoding enzymes of the peroxisomal ß-oxidation pathway by differentially modulating transactivation by PPARs.

2.3 MATERIALS AND METHODS

2.3.1 Plasmids

The luciferase reporter plasmids pCPS*luc*, pHD(X3)*luc* and pAOX(X2)*luc* and the effector plasmids expressing the full-length cDNAs for rat PPAR and human RXRα have been described previously (Marcus et al., 1993; Zhang et al., 1993). pCPS*luc* is a luciferase reporter plasmid containing the minimal promoter (a 600 nucleotides region from upstream of the transcription start site) for the carbamoyl-phosphate synthetase gene. The pHD(X3)*luc* construct was made by inserting three tandem copies of the sequence 5'-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA and its complement 5'-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAG corresponding to -2956 to -2919 of the HD gene into the unique *Bam*HI site of pCPS*luc*. The pAOx(X2)*luc* reporter plasmid contains two tandem copies of the sequence 5'-gatCCTTTCCCGAACGTGACCTT-TGTCCTGGTCCCCTTTTGCT and its complement 5'-gatctAGCAAAAGGGGACC-AGGACAAAGGTCACGTTCGGGAAG which encompasses the AOx-PPRE between -583 and -544 of the rat AOx gene. Nucleotides shown in lower case at the ends of the oligonucleotides were added to provide cohesive *Bam*HI-*Bgl*II ends at the 5' and 3' termini,

respectively. The rat PPAR cDNA in pBluescript II SK(+)(Stratagene) was provided by D. Noonan (Ligand Pharmaceuticals, San Diego) and was excised as a 2.6 Kbp *SpeI/EcoR*V fragment and inserted between the *XbaI/EcoR*I sites of pRC/CMV (Invitrogen). The human RXRα construct pSKXR3-1 was provided by R. Evans (The Salk Institute, La Jolla) and a 1.8 Kbp *EcoR*I fragment containing the cDNA was removed and inserted into the *EcoR*I site of the eukaryotic expression vector pSG5 (Stratagene). The β-galactosidase expression vector pCH110 (Amersham-Pharmacia Biotech) was included in transfections as an internal control for transfection efficiency. The plasmid for expression of rat HNF-4 was kindly provided by F.M. Sladek (University of California, Riverside) and contains the HNF-4 cDNA within a 3.0 Kbp fragment cloned into the *Bam*HI site of pSG5 (Sladek et al., 1990).

2.3.2 Transfections and Measurement of Luciferase Activity

All transfections were carried out in African green monkey kidney BSC40 monolayer cells using a modification of the calcium phosphate method (Ausubel, 1992; Zhang et al., 1993). Cells at 60-80% confluence were maintained in phenol-red free DMEM medium supplemented with 5% charcoal-stripped fetal bovine serum, 1% penicillin/streptomycin (Gibco BRL) and 4% L-glutamine (Gibco BRL), for 24 hours prior to and during transfection. Equal volumes of a 0.125 mM calcium chloride/DNA solution and a 2X Hepes-buffered saline solution (280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$, 50 mM Hepes (pH 7.12)) were combined and incubated at room temperature for 30 minutes. During this time, fresh transfection media containing either 0.1 mM of the peroxisome proliferator Wy-14,643 (in dimethyl-sulfoxide) or an equivalent volume of dimethyl-sulfoxide was added to the cells.

The DNA/calcium phosphate co-precipitate was divided into 1 mL aliquots and added dropwise to 10 cm plates. Transfections contained 5 μg of either the pHD(X3)*luc* or pAOx(X2)*luc* reporter gene construct, and where indicated, 2 μg of PPARα/RC/CMV, 2 μg of RXRα/SG5 and varying amounts of rHNF-4/SG5. The β-galactosidase expression vector (2 μg) was included in transfections and effector plasmid dosage was kept constant by addition of appropriate amounts of the corresponding empty vectors. Total DNA was kept at 20 μg per plate by addition of sonicated salmon sperm DNA (Sigma). Fresh transfection media containing Wy-14,643 or dimethyl-sulfoxide was added 16 hours post-transfection, and cells were incubated for an additional 24 hours. Cell harvesting, lysate preparation and quantification of luciferase activity were carried out as described previously (Marcus et al., 1993; Miyata et al., 1993; Zhang et al., 1993). Briefly, the cells were washed with phosphate-buffered saline, scraped from plates in 1 mL of phosphate-buffered saline and pelleted by centrifugation for 30 seconds. The supernatant was aspirated and cells were resuspended in 100 μL of 1X lysis buffer (2 mM CDTA, 1% (w/v) Triton X-100, 2 mM dithiothreitol, 10% (w/v) glycerol and 25 mM Tris-phosphate (pH7.8)). The lysates were prepared by intermittant vortexing for 10 minutes followed by brief centrifugation to remove cell debris. Aliquots of the supernatants were measured for luciferase activity by addition of reconstituted luciferase assay buffer and quantitation with a luminometer (Bio-Orbit, model 1523). The reconstituted luciferase assay buffer contains 0.53 mM ATP, 0.47 mM luciferin (Sigma), and 0.27 mM co-enzyme A (lithium salt, Sigma) in 10 mM dithiothreitol/25 mM glycylglycine (pH 7.8) added to basic luciferase assay buffer (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol (pH

7.8)).

2.3.3 In Vitro Transcription/Translation

Transcription and translation of cDNAs encoding PPARα, RXRα and HNF-4 were performed using the commercially available TNT T7 coupled rabbit reticulocyte lysate system according to manufacturer's protocol (Promega). Translation products were labelled with L-[^{35}S]methionine and were analyzed on 15% SDS-polyacrylamide gels. Synthesis of proteins used in electrophoretic mobility shift analysis (EMSA) was carried out in parallel with unlabelled methionine.

2.3.4 Electrophoretic Mobility Shift Analysis

H4IIEC3 nuclear extracts were prepared following Dignam's method (Dignam et al., 1983), except that following cell harvesting, cells were incubated on ice in buffer A for 10 minutes and subsequently lysed in buffer A supplemented with 0.5% NP-40. The nuclei were then collected by centrifugation at 2200 xg, and extracts were processed according to Dignam's method (Dignam et al., 1983). EMSA was performed with oligonucleotide probes corresponding to the HD-PPRE (5'-CCTCTCCTTTGACCTATTGAACTATTACCT-ACATTTGA and its complement), the AOx-PPRE (5'-CCTTTCCCGAACGTGACCTTTGT-CCTGGTCCCCTTTTGCT and its complement), and the M4-PPRE (5'-CCTCTCCTTattttaATTGAACTATTACCTACATTTGA and its complement (Miyata et al., 1993; Zhang et al., 1993). Underlined sequences indicate the locations of TGACCT-like motifs. M4-PPRE is a mutant version of the wild-type HD-PPRE

in which the first TGACCT-like repeat is mutated as indicated by the nucleotides in lower case. Complementary oligonucleotides were annealed and end-labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. 2 to 3 µl of programmed lysate was incubated with 0.2 pmol of labeled probe at 25°C in a final reaction volume of 15 µl containing 7 mM Hepes (pH 7.9), 140 mM NaCl, 1 mM EDTA, 7% (v/v) glycerol, 4 µg bovine serum albumin, 4 µg nonspecific competitor DNA (poly dI•dC and sonicated salmon sperm DNA, 1:1 weight ratio), 150 µM phenylmethylsulfonyl fluoride and 0.2 mM dithiothreitol. The total amount of reticulocyte lysate in each reaction was kept constant by addition of unprogrammed lysate. Where indicated, 1µl of preimmune serum or 1 µl of anti-rat HNF-4 serum was added to the binding reactions, which were then preincubated for 5 min prior to the addition of probe. Binding reactions were analyzed by electrophoresis at 4°C on prerun 3.5% polyacrylamide gels (30:1 acrylamide/N',N'-methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer, followed by autoradiography.

2.4 RESULTS

2.4.1 HNF-4 Interacts with the AOx- and HD-PPREs

Both the AOx-PPRE and the HD-PPRE contain two direct repeats of the TGACCT-related half site separated by a single nucleotide (denoted DR1) (Dreyer et al., 1992; Osumi et al., 1991; Zhang et al., 1993). The HD-PPRE contains an additional upstream direct repeat separated by a 2 nucleotide spacing (DR2) that is necessary for peroxisome proliferator-

responsiveness (Miyata et al., 1993). Fig. 2-1 compares the sequences of the AOx- and HD-PPREs with the HNF-4 consensus binding site (Sladek et al., 1990). The HNF-4 consensus binding sequence matches the AOx-PPRE in 11 of 12 positions, almost overlapping the DR1 repeat. The best match of the HNF-4 consensus binding sequence within the HD-PPRE is 8 of 12 nucleotides encompassing the entire first repeat and part of the second repeat of the HD-PPRE. This high degree of similarity led us to examine whether HNF-4 is among the rat liver nuclear proteins that bind to the AOx- and HD-PPREs.

As shown in Fig. 2-2, incubation of labelled AOx- (lane a) and HD-PPRE (lane d) probes with nuclear extracts prepared from H4IIEC3 cells, a rat hepatoma cell line responsive to peroxisome proliferators, resulted in the formation of a major protein/DNA complex in each case. We have previously demonstrated that PPAR, RXR, and COUP-TF are among the repertoire of DNA-binding proteins present in hepatoma cells that are capable of binding to both the AOx- and HD-PPREs (Miyata et al., 1993). We used antiserum to rat HNF-4 to determine whether HNF-4-like proteins were also present in these protein/DNA complexes. As shown in Fig. 2-2, inclusion of antiserum specific to HNF-4 (lane c) but not preimmune serum (lane b) led to the formation of a supershifted complex (arrowhead) and a corresponding decrease in the signal intensity of the major protein/DNA complex formed with the AOx-PPRE probe, indicating that HNF-4, or HNF-4-related proteins, present in this extract interact with the AOx-PPRE. A significant fraction of the AOx-PPRE/protein complex could be supershifted with the anti-HNF-4 serum, indicating that HNF-4 is a major component of the protein complexes formed on this element. In similar experiments carried out with the HD-PPRE, the generation of a supershifted complex with anti-HNF-4 serum was

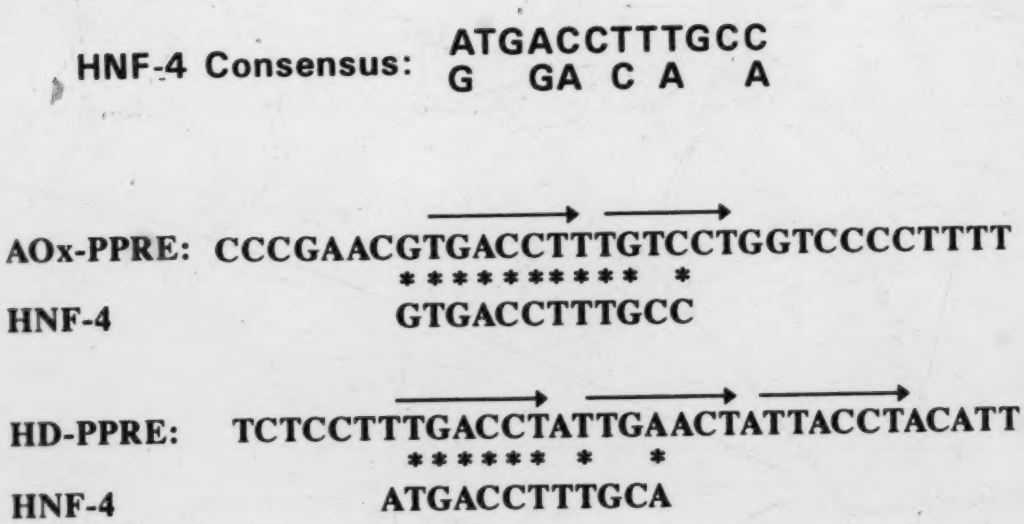


Figure 2-1. Comparison of the AOx-PPRE and the HD-PPRE to the consensus HNF-4 binding site. The consensus HNF-4 binding site (Sladek et al., 1990) is shown at top and is compared to the AOx- and HD-PPREs. The arrows indicate the TGACCT-related repeat motifs.

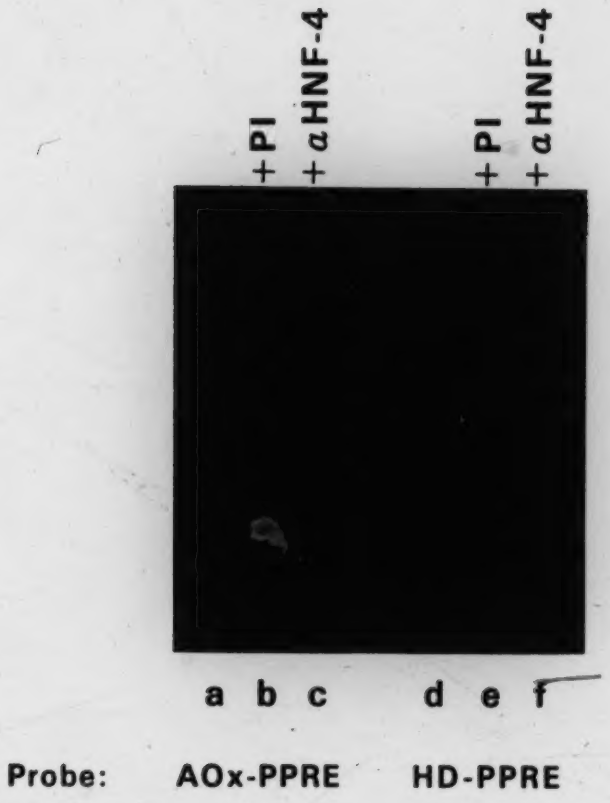


Figure 2-2. Endogenous HNF-4 related proteins of rat hepatoma H4IIEC3 cells bind to the AOx- and HD-PPREs. Electrophoretic mobility shift assays were performed with ^{32}P-labeled AOx-PPRE probe or HD-PPRE probe, as indicated at bottom. Probes were incubated with rat hepatoma H4IIEC3 nuclear extract alone (lanes a and d) or with nuclear extract supplemented with preimmune serum (+PI, lanes b and e) or anti-rat HNF-4 serum (+αHNF-4, lanes c and f). The arrowheads in lanes c and f indicate the supershifted complexes observed with anti-HNF-4 serum. This supershifted complex is readily evident with the AOx-PPRE probe (lane c) but is barely visible with the HD-PPRE probe (lane f). The band indicated by the open arrowhead in lane e is a nonspecific complex generated by a factor present in the preimmune serum that has a higher affinity for the HD-PPRE probe than for competitor DNA or for the AOx-PPRE probe.

at the limit of detection (arrowhead, lane f). The above results suggest that HNF-4 or HNF-4-related proteins present in extracts of rat liver hepatoma cells bind to the AOx-PPRE and, with much lower affinity, to the HD-PPRE. The differences in affinities correlate with the degree of relatedness of the respective PPREs to the HNF-4 consensus binding sequence.

To determine if HNF-4 could bind directly to the AOx- and HD-PPREs, we transcribed and translated rat HNF-4 *in vitro* from its cDNA and used the *in vitro* synthesized protein in mobility shift assays. As shown in Fig. 2-3, *in vitro* synthesized HNF-4 bound strongly to the AOx-PPRE probe (lane b) and more weakly to the HD-PPRE probe (lane d). The binding observed with both the AOx- and HD-PPREs was sequence specific, as determined by using mutant oligonucleotides as probes and competitors. For example, HNF-4 was unable to bind to a mutant HD-PPRE probe (M4) in which the second TGACCT repeat was mutated (Fig. 2-3, compare M4, lane f to HD, lane d), indicating that the first direct repeat in the HD-PPRE, which encompasses the HNF-4 consensus sequence, is required for binding to HNF-4. We have previously shown that luciferase reporter constructs containing the M4 mutant HD-PPRE have a reduced response to peroxisome proliferators *in vivo*, yet PPAR/RXR heterodimers could still efficiently bind to this element *in vitro* (Miyata et al., 1993). HNF-4 and PPAR/RXR may therefore have overlapping recognition sequences on the HD-PPRE, similar to what is observed with COUP-TF (Miyata et al., 1993). This finding would be consistent with the overlapping sequence identity between the middle repeat of the HD-PPRE and the HNF-4 consensus binding site (Fig. 2-1). It is not yet known whether all three receptors can occupy this site simultaneously.

Figure 2-3. HNF-4 synthesized *in vitro* binds to the AOx- and HD-PPREs. Electrophoretic mobility shift assays were carried out with the indicated labeled probes in the absence (-) or presence (+) of *in vitro* synthesized rat HNF-4. The arrowheads in lanes b and d indicate the HNF-4 protein/DNA complexes. The M4 probe used in lanes e and f is a mutant HD-PPRE in which the first TGACCT-like repeat is disrupted.

2.4.2 HNF-4 Represses PPARα-dependent Induction from the AOx-PPRE both in the Presence and Absence of a Potent Peroxisome Proliferator

To examine the consequences of ectopic expression of HNF-4 on transcription from the AOx- and HD-PPREs, we carried out transient transfection assays with luciferase reporter gene constructs containing the minimal carbamoyl phosphate synthetase promoter linked to either 2 tandem copies of the minimal AOx-PPRE (pAOx(X2)*luc*) or 3 tandem copies of the minimal HD-PPRE (pHD(X3)*luc*) (Zhang et al., 1993). The effector plasmids that were used expressed full-length cDNAs encoding rat PPARα, human RXRα, and rat HNF-4. BSC40 cells were used in the transient transfection assays, since efficient induction by peroxisome proliferators in this cell line requires the cotransfection of both PPAR and RXR (Miyata et al., 1993).

Cotransfection of pAOx(X2)*luc* with PPARα and RXRα expression vectors resulted in a 5- to 10-fold induction in activity of the reporter gene, as we have previously demonstrated (Fig. 2-4)(Marcus et al., 1993). This PPARα-mediated induction is independent of exogenously added peroxisome proliferators and likely results from the presence of endogenous PPAR and/or RXR activators present in these cells. Addition of the potent peroxisome proliferator Wy-14,643 resulted in a 20-fold stimulation of activity over basal levels. This stimulation required the presence of both PPARα and RXRα. Expression of HNF-4 during the transient transfection resulted in inhibition of both the PPARα-dependent/Wy-14,643-independent induction and the PPARα-dependent/Wy-14,643-dependent induction. Inhibition was dose-dependent under both circumstances. Inclusion of 4 μg of the HNF-4 expression plasmid reduced PPARα-dependent activation to the basal

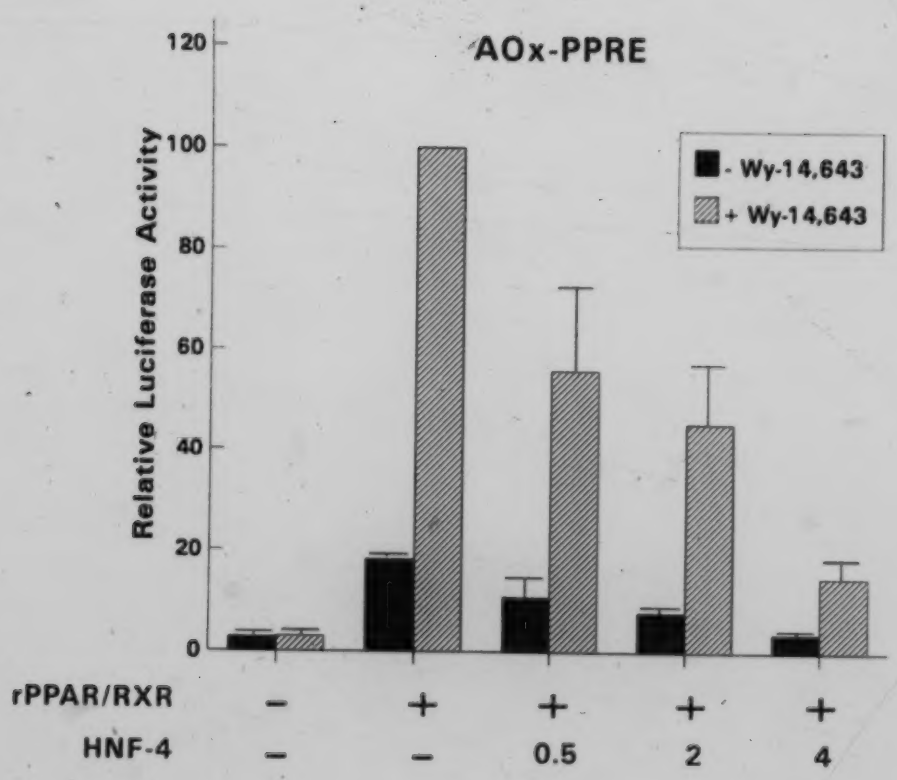


Figure 2-4. HNF-4 antagonizes rPPAR-mediated induction of an AOx-PPRE reporter gene. pAOx(X2)*luc* was transfected into BSC40 cells in the presence or absence of the peroxisome proliferator Wy-14,643, along with a constant amount of rPPARα and RXRα expression plasmids and increasing amounts of rat HNF-4 expression plasmid (in μg), as indicated. Transfections were carried out in duplicate and repeated a minimum of 3 times. The values shown (± standard error of the mean) are normalized to the value obtained from Wy-14,643-treated cells cotransfected with rPPAR and RXRα expression plasmids, which was taken as 100%.

levels observed with pAOx(X2)*luc* alone. Similarly, peroxisome proliferator-dependent induction was almost completely inhibited in the presence of 4 μg of HNF-4 expression plasmid. HNF-4-dependent inhibition was specific for the PPARα-mediated response, since the basal level expression of pAOx(X2)*luc* was not significantly affected by cotransfection with increasing amounts of HNF-4 expression plasmid (Fig. 2-6) These findings demonstrate that HNF-4 antagonizes induction from the AOx-PPRE by PPARα, both in the absence and in the presence of an exogenously added peroxisome proliferator.

2.4.3 HNF-4 Potentiates Activity of PPARα on the HD-PPRE in the Presence of a Peroxisome Proliferator

Transfection experiments like those carried out with the AOx-PPRE reporter gene construct were carried out with the HD-PPRE reporter gene construct. Cotransfection of pHD(X3)*luc* with both PPARα and RXRα expression plasmids gave a 5- to 10-fold peroxisome proliferator-independent induction and a 50-fold peroxisome proliferator-dependent induction of reporter gene activity over basal levels (Fig. 2-5). As seen with the AOx-PPRE reporter gene construct, inclusion of HNF-4 during transient transfection resulted in inhibition of the peroxisome proliferator-independent activation mediated by PPARα/RXRα from the HD-PPRE. However, HNF-4-mediated inhibition was less efficient with the HD-PPRE than with the AOx-PPRE, causing only a 50% inhibition of activation with 4 μg of HNF-4 expression plasmid as compared to a control transfection in the absence of HNF-4. Surprisingly, when Wy-14,643 was included in the transfections, HNF-4 significantly stimulated the induction response of the HD-PPRE. Cotransfection with 4 μg

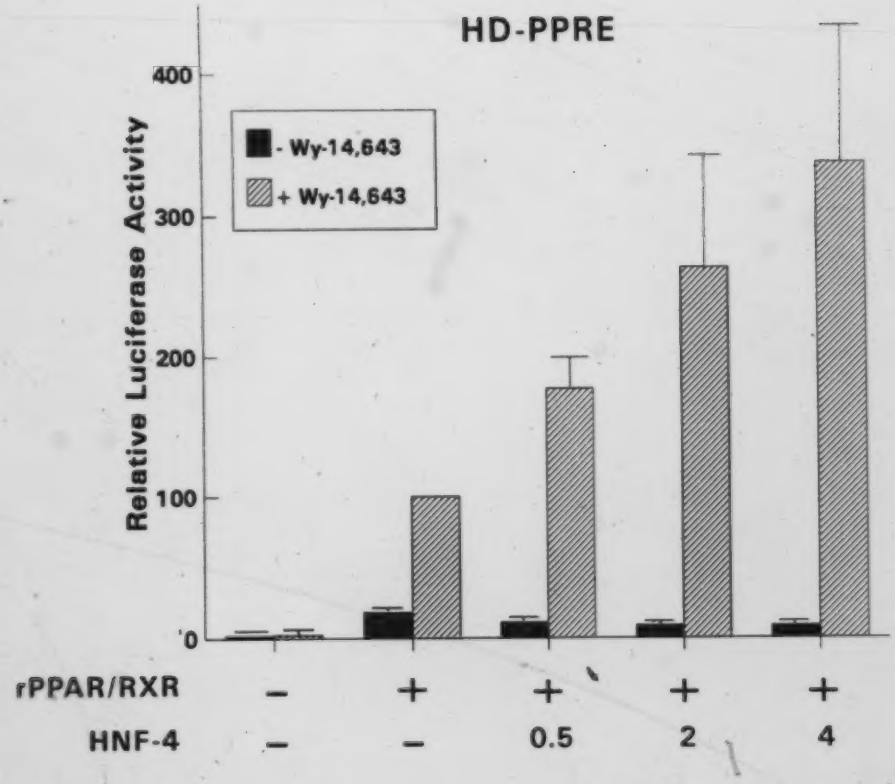


Figure 2-5. HNF-4 differentially modulates the function of rPPARα on the HD-PPRE. Cotransfection of pHD(X3)*luc* with various expression plasmids into BSC40 cells in the presence or absence of Wy-14,643 and luciferase activity measurements were performed as described in Fig. 2-4. The results demonstrate that HNF-4 antagonizes rPPARα function in the absence of Wy-14,643 but potentiates rPPARα activity in the presence of Wy-14,643.

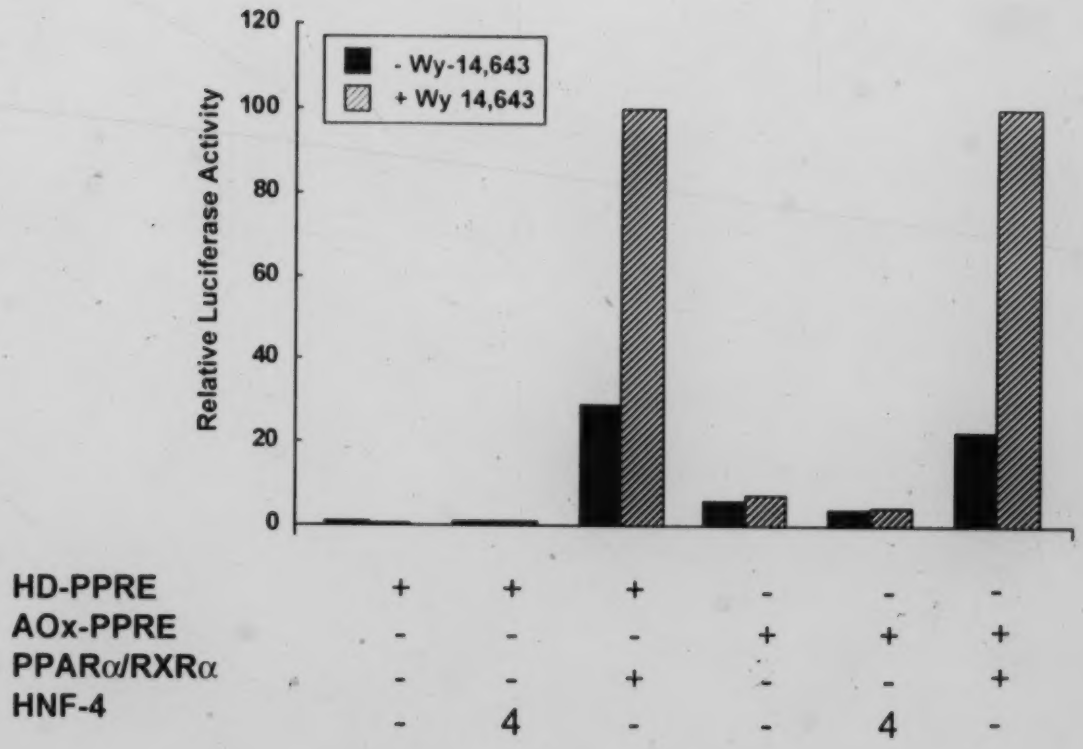


Figure 2-6. HNF-4 alters transactivation from the AOx- and HD-PPREs in the presence of rPPARα and RXRα. BSC40 cells were co-transfected as before with pAOx(X2)*luc* or pHD(X3)*luc* reporter plasmids and with rPPAR and RXRα (2 μg each) or with HNF-4 (4 μg) expression plasmids as indicated. Co-transfections were performed with or without the peroxisome proliferator Wy-14,643. Values are presented relative to the value obtained for cells co-transfected with rPPAR and RXRα in the presence of Wy-14,643 (taken as 100%). The values represent the average of three independent transfections carried out in duplicate.

of HNF-4 expression plasmid resulted in a 2- to 3-fold potentiation of the PPARα-mediated response to the presence of this peroxisome proliferator. Both repression and stimulation by HNF-4 required the presence of PPARα and RXRα, since HNF-4 alone had no significant effects on the basal level expression of the HD-PPRE reporter gene construct (Fig. 2-6). Therefore, in contrast to the results seen with the AOx-PPRE, HNF-4 cooperates with PPARα to stimulate transcription of the HD-PPRE reporter gene construct, but only in the presence of a peroxisome proliferator. Because HNF-4 both repressed the PPARα-mediated response in the absence of drug and increased the response in the presence of drug, the peroxisome proliferator-dependent induction ratio was effectively increased from 5-fold to approximately 35-fold.

2.4.4 HNF-4 Does Not Cooperate with RXRα or PPARα for DNA Binding

PPARα heterodimerizes with RXRα and binds cooperatively to both the AOx- and HD-PPREs *in vitro* (Fig. 2-7, compare lanes b and c with lanes e)(Marcus et al., 1993). In contrast, HNF-4 has been shown to bind to cognate response elements exclusively as a homodimer, and there is no evidence that HNF-4 heterodimerizes with other members of the nuclear hormone receptor family (Sladek et al., 1990). Nevertheless, we explored the possibility that the effects of HNF-4 on PPARα-dependent induction of transcription *in vivo* was mitigated by cooperative interactions with PPAR and/or RXRα by mobility shift analyses with *in vitro* translated receptor proteins. As shown in Fig. 2-7, the binding of HNF-4 to the AOx-PPRE or the HD-PPRE was not affected by the presence of RXRα or PPARα and *vice versa* (compare lanes d to lanes f and g, respectively). Therefore, the ability of HNF-4 to

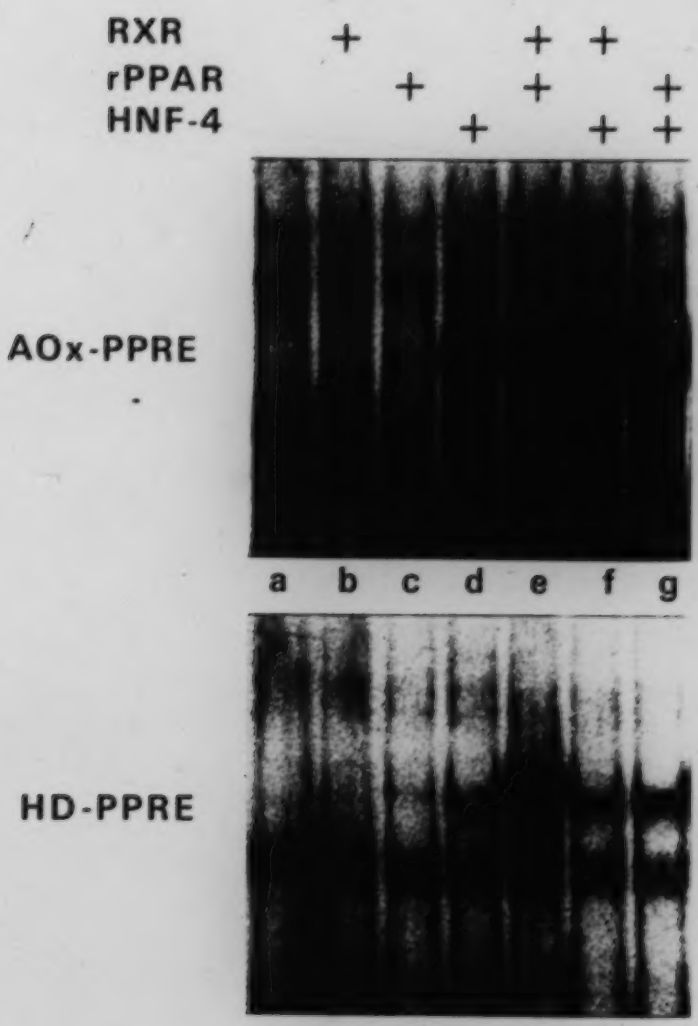


Figure 2-7. HNF-4 does not cooperate with rPPARα or RXRα for DNA binding. *In vitro* translated human RXRα, rPPARα and rat HNF-4 were incubated singly or in pairwise combinations, as indicated, with labeled AOx-PPRE probe (upper panel) or HD-PPRE probe (lower panel) and analyzed by electrophoretic mobility shift assay. Lanes a contained probes incubated with unprogrammed reticulocyte lysate. Arrow, nonspecific complex formed between the HD-PPRE probe and reticulocyte lysate.

modulate PPAR function apparently is not the result of cooperative DNA binding by HNF-4 with either RXRα or PPARα.

2.5 DISCUSSION

The results presented herein demonstrate that HNF-4 differentially modulates the functioning of the peroxisome proliferator-activated receptor on specific PPREs, suggesting that this nuclear hormone receptor plays a role in the regulation of expression of genes encoding peroxisomal ß-oxidation enzymes. HNF-4 has been shown to act as a cell-restricted, positive regulator of genes whose products are involved in several metabolic pathways (Chen et al., 1994; Ladias et al., 1992; Sladek et al., 1990). Our findings are the first demonstration that HNF-4 can both activate and repress specific target genes involved in lipid metabolism through a common response element.

HNF-4 was a major component of the protein/DNA complex formed between the AOx-PPRE and rat liver hepatoma nuclear extracts, and HNF-4 synthesized *in vitro* interacted strongly with the AOx-PPRE. In transient transfection assays, HNF-4 competitively inhibited PPARα-dependent transactivation of a reporter gene linked to the AOx-PPRE, both in the presence or the absence of the peroxisome proliferator, Wy-14,643. The mechanism by which HNF-4 antagonizes PPARα functioning is not known at present, but one scenario is that HNF-4 can compete directly with PPARα/RXR heterodimers for binding to the AOx-PPRE. This explanation would be consistent with the near perfect match between the consensus HNF-4 binding site and part of the AOx-PPRE and with the fact that the region of homology between the two sequences almost completely overlaps the two

TGACCT DR1 repeats that are essential for PPARα/RXRα binding to the AOx-PPRE. HNF-4 was unable to bind cooperatively with either PPARα or RXRα, implying that competition for heterodimerization partners is probably not involved in the observed inhibition of activation from the AOx-PPRE. However, it remains possible that under physiological conditions, non-DNA binding heteromers could form between HNF-4 and PPARα or RXRα, thereby leading to inhibition of the PPARα-mediated response via indirect mechanisms.

Inhibition of activation from the AOx-PPRE by HNF-4 could result from the absence of an appropriate ligand for HNF-4 in the BSC40 cells used in the transfection assays. It has been shown that, depending on the nature of the response element, the thyroid receptor can function as a repressor in the unliganded state but as an activator when associated with its cognate hormone (Damm et al., 1989; Graupner et al., 1989; Sap et al., 1989). The repression observed with the thyroid receptor in the absence of its ligand is thought to be mediated via an active intrinsic silencing domain that may directly inhibit the formation of the functional preinitiation complex (Baniahmad et al., 1992; Fondell et al., 1993). There is no evidence as of yet that HNF-4 possesses an active transcriptional silencing function or even that a ligand exists for this receptor.

The results obtained with the HD-PPRE are even more intriguing than those obtained with the AOx-PPRE. HNF-4 had a repressive effect (50%) on the PPARα-dependent activation of the HD-PPRE in the absence of exogenously added peroxisome proliferator. However, in the presence of Wy-14,643, HNF-4 cooperated with PPARα/RXRα to provoke a significant stimulation of transcription. Therefore, under conditions of peroxisome proliferator administration, HNF-4 had diametric effects on the PPARα-mediated, peroxisome

proliferator responsiveness of the AOx-PPRE *vis-à-vis* the HD-PPRE. It is unclear whether the effects of HNF-4 on activation from the HD-PPRE are mitigated directly through binding to the HD-PPRE or indirectly, for example, through interaction with, or modulation of, auxiliary coregulators involved in peroxisome proliferator signalling or transcriptional control.

HNF-4 synthesized *in vitro* or present endogenously in rat hepatoma cells had only a weak affinity for the HD-PPRE, although its binding to the HD-PPRE was specific for the sequence of this element. The structure of the HD-PPRE is more complex than that of the AOx-PPRE. The HD-PPRE consists of three TGACCT-related half-sites separated by 2 nucleotides and 1 nucleotide, respectively. The integrity of all three repeats, as well as the spacing between them, are necessary for efficient peroxisome proliferator responsiveness *in vivo*; however, only the DR1 repeats are necessary for PPARα/RXRα-binding *in vitro* (Miyata et al., 1993). The region within the HD-PPRE that most closely matches the HNF-4 consensus sequence contains the first direct repeat. We have shown that this repeat is necessary for HNF-4 binding (Fig. 2-3). It is conceivable that binding of HNF-4 to the first repeat might interfere with binding of PPARα/RXR to the HD-PPRE, leading to the inhibitory effects on activation observed in transient transfections. Activation of PPARα by exogenously added peroxisome proliferators may serve to alter the binding affinity or conformation of the PPARα/RXRα heterodimer specifically on the HD-PPRE, thereby allowing cooperative interactions with HNF-4 and consequently potentiation of induction. We have previously shown that the first repeat in the HD-PPRE is also required for binding to COUP-TF1, an orphan receptor that antagonizes peroxisome proliferator-responsiveness *in vivo* (Miyata et al., 1993). Therefore, the additional repeat element present in the HD-

PPRE compared to the AOx-PPRE may allow more complex strategies of regulation for the HD gene.

It is becoming increasingly apparent that PPARs play a central role in regulating lipid metabolic pathways. More recently, PPARs have been implicated in cellular differentiation and proliferation (Chawla and Lazar, 1994). Our findings suggest that HNF-4 can modulate PPAR function both positively and negatively, consequently resulting in differential regulation of at least two distinct peroxisome proliferator-responsive genes. These differential effects are conditional upon the structure of a particular target PPRE and the presence or absence of PPAR activators. This finding is interesting in light of the recent observation that HNF-4 activates transcription of the medium-chain acyl-CoA dehydrogenase gene (Carter et al., 1993). Medium-chain acyl-CoA dehydrogenase is the initial and rate-limiting enzyme of the mitochondrial fatty acid ß-oxidation pathway, which metabolizes short- and medium-chain length fatty acids in response to energy demands of the cell. In contrast, the peroxisomal ß-oxidation pathway preferentially uses long-chain fatty acids as substrates, and acyl-CoA oxidase is the initial and rate-limiting step of this pathway. Our finding that HNF-4 represses PPAR-mediated induction of the AOx gene suggests that HNF-4 may play a key role in coordinating and integrating the mitochondrial and peroxisomal ß-oxidation pathways through the bidirectional regulation of the respective rate-limiting enzymes. Similarly, the HNF-4-mediated, peroxisome proliferator-dependent upregulation of the activity of the HD gene, whose product catalyzes the second step in the peroxisomal ß-oxidation pathway, may represent an adaptive cellular response that primes the peroxisomal ß-oxidation pathway to respond rapidly to cellular oxidative demands under physiological conditions where repression

of the AOx gene is alleviated. HNF-4/PPAR cooperativity on the HD-PPRE may be relevant to the observation that HD accumulates to higher levels in the liver compared to the other two enzymes of the peroxisomal ß-oxidation pathway following administration of peroxisome proliferators (Reddy et al., 1986a). Our findings add an additional layer of complexity to peroxisome proliferator-signalling pathways and may begin to provide insight into how combinatorial interactions among distinct nuclear receptors and convergence of multiple signalling pathways integrate complex metabolic regulatory networks.

CHAPTER 3

Calreticulin Modulates the In Vitro DNA Binding But Not the In Vivo Transcriptional Activation by Peroxisome Proliferator-Activated Receptor/Retinoid X Receptor Heterodimers†

†A version of this chapter has been published. Winrow, C.J., Miyata, K.S., Marcus, S.L., Burns, K., Michalak, M., Capone, J.P. and Rachubinski. 1995. Mol. and Cell. Endocrinol. **111**:175-179. Used with permission from Elsevier Science.

3.1 ABSTRACT

Calreticulin is a ubiquitous calcium binding/storage protein found primarily in the endoplasmic reticulum. Calreticulin has been shown to inhibit DNA binding and transcriptional activation by glucocorticoid and androgen hormone receptors by binding to the conserved sequence KXFF(K/R)R, present in the DNA-binding domains of all known members of the steroid/nuclear hormone receptor superfamily. To determine whether calreticulin might be a general regulator of hormone-responsive pathways, we examined its effect on DNA binding *in vitro* and transcriptional activation *in vivo* by heterodimers of the peroxisome proliferator-activated receptor (PPAR) and the 9-*cis* retinoic acid receptor (RXRα). We show here that purified calreticulin inhibits the binding of PPARα/RXRα heterodimers and of other nuclear hormone receptors, to peroxisome proliferator-responsive DNA elements *in vitro*. However, overexpression of calreticulin in transiently transfected cultured cells had little or no effect on transactivation mediated by PPARα/RXRα. Therefore, while calreticulin inhibits the binding of both nuclear and steroid hormone receptors to cognate response elements *in vitro*, our findings suggest that calreticulin does not necessarily play an important role in the regulation of all classes of hormone receptors *in vivo*.

3.2 INTRODUCTION

Members of the steroid/nuclear hormone receptor superfamily play crucial roles in organismal development, differentiation and homeostasis. These receptors mediate physiological and cellular responses to extracellular and intracellular stimuli through the regulation of diverse gene networks. Steroid/nuclear hormone receptors are structurally related ligand-activated transcription factors that bind to cognate response elements upstream of target genes, conferring responsiveness to steroid hormones such as glucocorticoids, androgens and estrogen and to non-steroid hormones such as retinoids, thyroid hormone, and vitamin D3. These transcription factors are subject to a variety of post-transcriptional regulatory strategies that serve to modulate their subcellular distribution, abundance, DNA-binding capacity, and function. Therefore, steroid receptors like the glucocorticoid receptor (GR), estrogen receptor, progesterone receptor, and androgen receptor (AR) are found in multi-component cytoplasmic complexes with non-receptor cellular factors, including the heat shock proteins hsp90, hsp70 and hsp56. Ligand binding facilitates the dissociation of the receptor from this complex, with subsequent nuclear translocation and binding of the receptor to cognate response sites (Evans, 1988; Truss and Beato, 1993; Tsai and O'Malley, 1994).

Recently, calreticulin has been shown to modulate DNA binding and transcriptional activation by AR and GR (Burns et al., 1994; Dedhar et al., 1994). Calreticulin is a multifunctional Ca^{2+}-binding/storage, lumenal protein of the endoplasmic reticulum (Michalak et al., 1992) that has been shown to interact with the conserved amino acid sequence KLGFFKR of integrins (Rojiani et al., 1991). This sequence is similar to the sequence KXFF(K/R)R found in the conserved DNA-binding domain of members of the steroid/nuclear

hormone receptor superfamily (Burns et al., 1994; Laudet et al., 1992). Calreticulin was shown to bind to this sequence present in GR and AR, resulting in the inhibition of these receptors to bind to DNA *in vitro* and to activate transcription *in vivo* (Burns et al., 1994; Dedhar et al., 1994). These findings are surprising since calreticulin is localized predominantly to the endoplasmic reticulum, and therefore would not be expected to have access to nuclear-resident transcription factors. The exact mechanism responsible for calreticulin-mediated modulation of steroid receptor function *in vivo* remains unclear.

Since the consensus calreticulin binding site is conserved in all members of the steroid/nuclear hormone receptor superfamily, calreticulin might be considered to be a general regulator of all nuclear hormone-responsive pathways. GR and AR are found both in the cytoplasm and the nucleus and bind to their response elements as homodimers. Receptors for retinoic acid (RAR), 9-*cis* retinoic acid (RXRα), vitamin D3, thyroid hormone, and peroxisome proliferators (PPAR) constitute a functionally distinct subclass within the steroid/nuclear hormone receptor superfamily. These receptors are constitutively localized to the nucleus, do not form complexes with cytoplasmic factors, and can bind to DNA in the absence of ligand. Nuclear hormone receptors can bind to DNA as homodimers, although high-affinity DNA binding is generally manifested through heterodimerization with a common auxiliary cofactor such as RXR.

We explored the role of calreticulin on gene regulation by this class of nuclear hormone receptors by investigating its effects on the function of human RXRα and rat PPARα. RXR and PPAR bind as heterodimers to peroxisome proliferator-response elements (PPREs) and mediate transcriptional activation by peroxisome proliferators, a broad class of

xenobiotic compounds, and by selected fatty acids (Gearing et al., 1993; Gottlicher et al., 1992; Issemann and Green, 1990; Kliewer et al., 1992a; Marcus et al., 1993). We show that calreticulin inhibits the *in vitro* binding of PPAR/RXR heterodimers to the PPRE of the gene encoding fatty acyl-CoA oxidase (AOx), the first enzyme of the peroxisomal ß-oxidation cascade. However, in transiently transfected cultured cells, overexpression of calreticulin results in only marginal effects on the transcriptional activation of the AOx PPRE by PPAR/RXR. We conclude that while calreticulin can inhibit the DNA binding of both steroid and nuclear hormone receptors *in vitro*, it may not be physiologically important in the *in vivo* regulation of all members of this receptor superfamily.

3.3 MATERIALS AND METHODS

3.3.1 Plasmids, Cell Culture, and Transfection

The luciferase reporter plasmids pCPS*luc*, containing the minimal promoter of the rat carbamoyl phosphate synthetase gene, and pAOx(X2)*luc*, containing 2 copies of the minimal PPRE of the rat AOx gene (5'-CCTTTCCGAACGTGACCTTTGTCCTG-GTCCCCTTTTGCT), have been described in Chapter 2 (Zhang et al., 1993). The glucocorticoid response element (GRE) reporter plasmid MMTV-GRE*luc* (pJA358) has been reported (Burns et al., 1994). Plasmid vectors for *in vivo* expression and *in vitro* transcription/translation of rat PPARα, human RXRα, and human HNF-4 have been described (Marcus et al., 1993; Winrow et al., 1994). The expression plasmid for human COUP-TF1 contains the COUP-TF1 cDNA inserted as an *HindIII/Xba*I fragment into pRC/CMV (Miyata

et al., 1993). The expression vector pSVL-CRT was constructed by insertion of the cDNA encoding full-length rabbit skeletal muscle calreticulin into the plasmid pSVL (Pharmacia)(Burns et al., 1994). The plasmid VARO was used to express GR and was provided by K.R. Yamamoto (University of California, San Diego)(Pearce and Yamamoto, 1993). Transient transfection assays were carried out in BSC40 cells (Miyata et al., 1993) using various expression vectors as indicated in the figure legends. Luciferase activity was measured as described in Chapter 2. Where indicated, the peroxisome proliferator Wy-14,643 or dexamethasone was added to transfected cells at a final concentration of 0.1 mM and 1 μM, respectively (Marcus et al., 1993). The plasmid pCH110 (Pharmacia) containing the *lacZ* gene was included in transfections to normalize for the efficiency of transfection.

3.3.2 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were done with ^{32}P-labelled AOx-PPRE probe and receptors transcribed and translated *in vitro* from their respective cDNAs, as previously described in Chapter 2 (Miyata et al., 1993). Where indicated, 1 μg of purified calreticulin (Baksh et al., 1992) was added to the reactions prior to the addition of probe DNA.

3.4 RESULTS

3.4.1 Calreticulin Inhibits PPAR/RXR Binding to DNA

Calreticulin has been reported to interact with the DNA-binding domains of GR (Burns et al., 1994) and AR (Dedhar et al., 1994) via the conserved amino acid sequence

KXFF(R/K)R located between the two "zinc fingers" in these and other steroid receptors (Laudet et al., 1992). In this study we examined whether calreticulin could inhibit the cooperative DNA binding of PPAR/RXR heterodimers to the AOx-PPRE. Rat PPARα (hereafter called PPAR) and human RXRα (hereafter called RXR) contain the sequences KGFFRR and KGFFKR, respectively, in their DNA-binding domains (Fig. 3-1). An electrophoretic mobility shift assay was done with *in vitro* translated PPAR and RXR and a radiolabelled synthetic oligonucleotide probe corresponding to the AOx-PPRE. *In vitro* translated PPAR and RXR formed a specific protein/DNA complex on the AOx-PPRE (Fig. 3-2, *lane c*). PPAR and RXR do not bind individually to the AOx-PPRE (Gearing et al., 1993; Kliewer et al., 1992a; Marcus et al., 1993). Addition of purified calreticulin abolished the binding of the PPAR/RXR heterodimer to the AOx-PPRE (*lane f*). Calreticulin alone did not interact with the AOx-PPRE (*lane b*). COUP-TF1 and HNF-4, two additional nuclear hormone receptors, also form complexes as homodimers on the AOx-PPRE (Miyata et al., 1993; Winrow et al., 1994)(Fig. 3-2, *lanes d* and *e*, respectively). Complex formation by either receptor was completely abolished by addition of calreticulin (compare *lane d* to *lane g* and *lane e* to *lane h*). Therefore, calreticulin interacts *in vitro* with PPAR/RXR heterodimers and COUP-TF1 and HNF-4 homodimers, preventing their binding to the AOx-PPRE.

3.4.2 Calreticulin Shows Little or No Effect on Transactivation by PPAR/RXR In Vivo

Transient transfection assays were carried out to investigate the effects of calreticulin expression on transactivation mediated by PPAR/RXR *in vivo*. These experiments used the

PPARα	VHACEGC	**KGFFRR**	T I RL KLA
RXRα	VYSCEGC	**KGFFKR**	TVRKD LT
COUP-TF1	VSSSV I E	**QLFFVR**	LVGKT P I
HNF-4	ASSCDGC	**KGFFRR**	SVRKNHM
GR	VLTCGSC	**KVFFKR**	AVEGQHN
CRT binding site		**KXFF(K/R)R**	

Figure 3-1. The DNA-binding domains of nuclear hormone and steroid receptors contain sequences homologous to the calreticulin binding site. Portions of the DNA-binding regions of the nuclear hormone receptors rat PPARα, human RXRα, human COUP-TF1, and rat HNF-4 and of the steroid receptor human GR are shown, along with the consensus calreticulin (CRT) binding site. The amino acids highlighted in bold conform to the consensus calreticulin binding site.

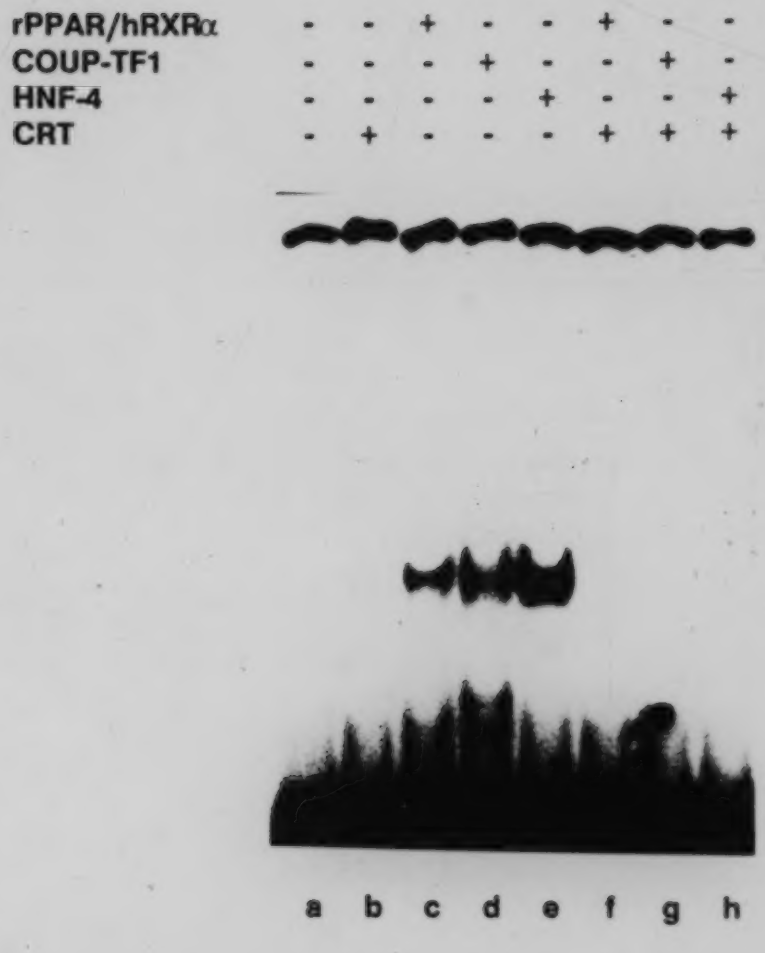
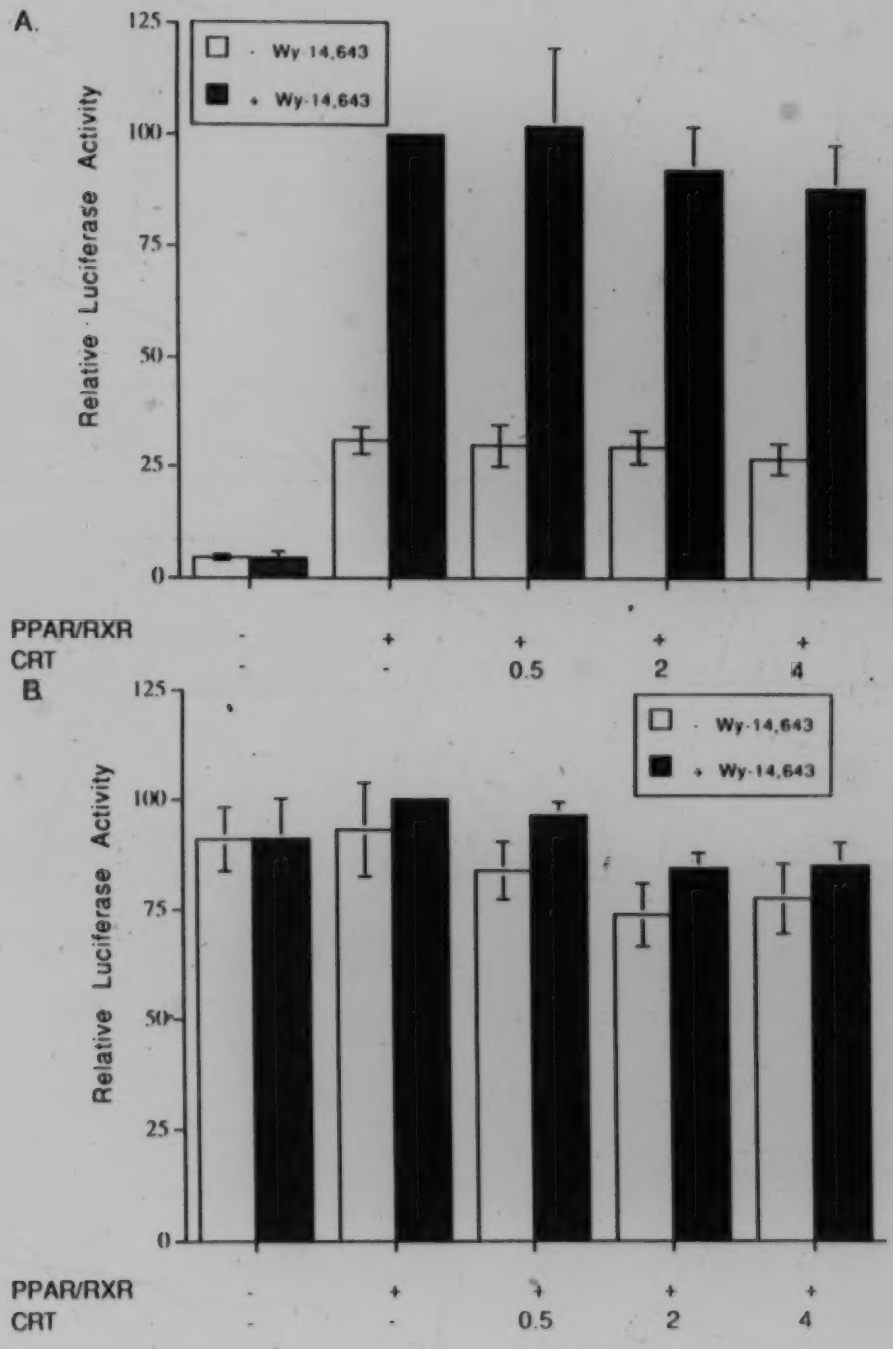


Figure 3-2. Calreticulin inhibits PPAR/RXR heterodimer binding to the AOx-PPRE *in vitro*. An electrophoretic mobility shift assay was performed with ^{32}P-labeled AOx-PPRE probe and *in vitro* translated PPAR and RXR (*lane c*). The AOx-PPRE probe was also incubated with *in vitro* translated COUP-TF1 (*lane d*) and HNF-4 (*lane e*). Incubation of the *in vitro* translated receptors with the AOx-PPRE probe was for 25 min at 28°C. Where indicated, 1 μg of purified rabbit calreticulin (CRT) was preincubated with *in vitro* translated PPAR/RXR (*lane f*), COUP-TF1 (*lane g*), and HNF-4 (*lane h*) for 15 min at 28°C, followed by incubation with the AOx-PPRE probe as described above. No complex was formed by the addition of unprogrammed reticulocyte lysate (*lane a*).

luciferase reporter gene plasmid construct pAOx(X2)*luc* containing 2 copies of the AOx-PPRE, which has been shown to be responsive to transactivation by PPAR/RXR (Marcus et al., 1993). In agreement with our earlier report (Marcus et al., 1993; Winrow et al., 1994), cotransfection of BSC40 cells with pAOx(X2)*luc* and plasmids expressing PPAR and RXR led to 6-fold and 20-fold increases in luciferase reporter gene activity over control transfections containing empty vectors in the absence and presence of the added peroxisome proliferator Wy-14,643, respectively (Fig. 3-3A). Addition of increasing amounts of the calreticulin expression plasmid pSVL-CRT to the transfections showed no significant effects on either the peroxisome proliferator-dependent or peroxisome proliferator-independent activation of the reporter gene construct by PPAR/RXR. The slight inhibition of transactivation (10 to 15%) observed with increasing amounts of pSVL-CRT is apparently nonspecific, as similar levels of inhibition were seen in control transfections carried out with pCPS*luc*, the parental reporter plasmid used to construct pAOx(X2)*luc* (Fig. 3-3B).

Given the dramatic inhibitory effect of calreticulin on the *in vitro* binding of PPAR/RXR to the AOx-PPRE (Fig. 3-2), it was surprising that calreticulin expression had very little or no effect on the PPAR/RXR-mediated *in vivo* activation of the pAOx(X2)*luc* reporter construct. Burns et al. (Burns et al., 1994) have shown that overexpression of calreticulin in mouse L cells results in a 50 to 80% inhibition in the GR-mediated expression of a luciferase reporter gene under the control of a MMTV-GRE promoter. Therefore, to insure that calreticulin was functionally expressed under the experimental conditions used in this study, we transfected BSC40 cells with the MMTV-GRE*luc* reporter plasmid pJA358, the VARO plasmid expressing GR, and the calreticulin expression vector pSVL-CRT. The

Figure 3-3. Calreticulin fails to repress the *in vivo* transactivation from the AOx-PPRE by PPAR/RXR. (*A*). pAOx(2X)*luc* was transfected into BSC40 cells along with effector plasmids expressing PPAR, RXR, and calreticulin (CRT). Transfections contained 5 μg of the reporter plasmid pAOx(X2)*luc* and, where indicated, 2 μg of hRXRα-expression plasmid, 2 μg of rPPARα-expression plasmid, and varying amounts (in μg) of the calreticulin-expression plasmid pSVL-CRT. Control transfections contained the corresponding empty vectors. Cells were grown in media that did or did not contain 0.1 mM of the peroxisome proliferator, WY-14,643. Cells were harvested 48-h post-transfection, and luciferase activity was determined. The values shown are relative activities from three independent transfections done in duplicate and were normalized to the value obtained for WY-14,643-treated cells cotransfected with rPPAR and hRXRα expression plasmids, which was taken as 100%. (*B*). Transfection was done as in (*A*), except that the reporter plasmid was pCPS*luc*. The values shown are relative activities from three independent transfections done in duplicate and were normalized as in (*A*).


A.
Relative Luciferase Activity
125
100
75
50
25
0
- Wy-14,643
+ Wy-14,643
PPAR/RXR
CRT
- + + + +
- - 0.5 2 4
B.
Relative Luciferase Activity
125
100
75
50
25
0
- Wy-14,643
+ Wy-14,643
PPAR/RXR
CRT
- + + + +
- - 0.5 2 4

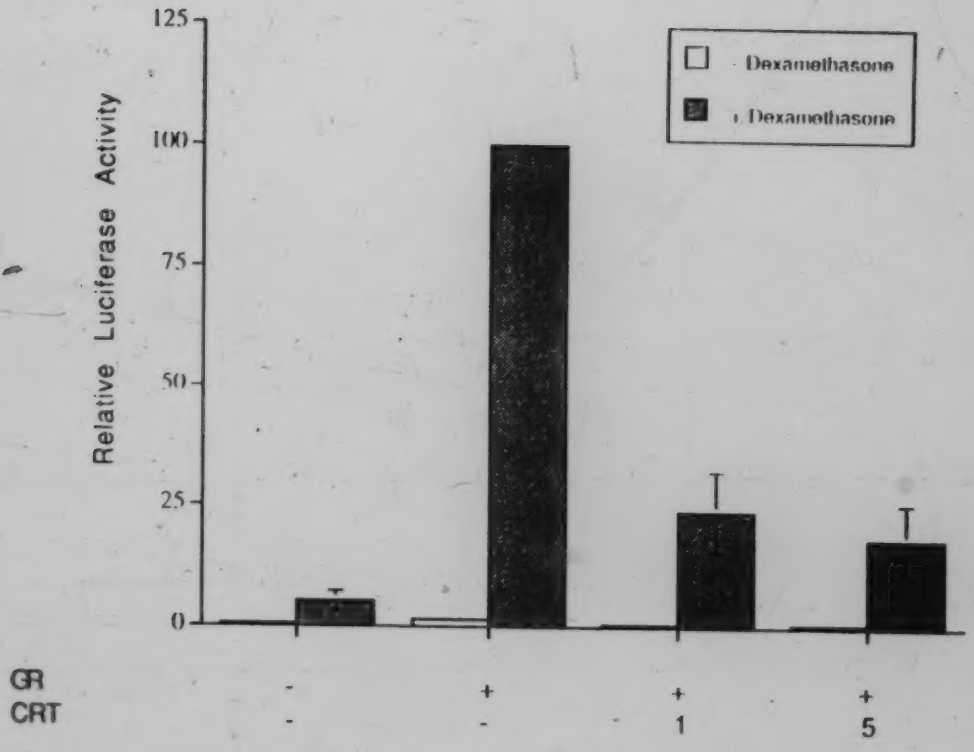


Figure 3-4. Calreticulin represses transactivation from the GRE by GR. The GRE reporter plasmid pJA358 was transfected into BSC40 cells along with effector plasmids expressing GR and calreticulin. Transfections contained 5 μg of the reporter plasmid pJA358 and, where indicated, 2 μg of the GR-expression plasmid VARO and varying amounts (in μg) of the calreticulin-expression plasmid pSVL-CRT. Control transfections contained the corresponding empty vectors. Cells were grown in media that did or did not contain 1 μM dexamethasone. Cells were harvested 48h post-transfection, and luciferase activity was determined. The values shown are relative activities from three independent transfections done in duplicate and were normalized to the value obtained for dexamethasone-treated cells transfected with the GR-expression plasmid VARO, which was taken as 100%.

presence of the VARO plasmid increased luciferase activity from pJA358 70-fold upon addition of dexamethasone (Fig. 3-4). This dexamethasone-dependent activation of the reporter gene was inhibited 50 to 90% when the calreticulin expression vector pSVL-CRT was included in the transfection (Fig. 3-4). Therefore, calreticulin inhibits GR-mediated stimulation of gene expression in BSC40 cells, as previously demonstrated in mouse L cells (Burns et al., 1994). This finding suggests that transfection of BSC40 cells with pSVL-CRT led to expression of functional calreticulin which, while capable of inhibiting the transcriptional activation function by GR, had little or no effect on transcriptional activation by PPAR/RXR.

3.5 DISCUSSION

We have shown that calreticulin inhibits the *in vitro* DNA binding of the nuclear hormone receptors PPAR and RXR, as well as COUP-TF1 and HNF-4, to a cognate response element, as was previously observed with GR and AR. However, in contrast to the results observed with GR and AR (Burns et al., 1994; Dedhar et al., 1994), overexpression of calreticulin in transiently transfected cells had little or no effect on PPAR/RXR-mediated activation of gene expression.

For calreticulin to play a physiologically relevant role in the regulation of steroid and nuclear hormone receptors, sufficient calreticulin must gain access to the cytoplasm and/or the nucleus (Bleackley et al., 1995). However, calreticulin is localized primarily to the lumen of the endoplasmic reticulum, since it is synthesized with a signal sequence and contains a KDEL retention sequence (Bleackley et al., 1995; Michalak et al., 1992).

Immunofluorescence studies have suggested that some calreticulin can be found in the nuclear envelope and the cytoplasm in some cell types, perhaps in amounts sufficient to affect steroid receptor function (Michalak et al., 1992; Nash et al., 1994; Opas et al., 1991). However, calreticulin did not inhibit PPAR/RXR function *in vivo*. The lack of a pronounced effect of calreticulin expression on PPAR/RXR function *vis-à-vis* GR and AR function may be due to differences in their pathways of activation. Calreticulin may inhibit the function of GR and AR at some step unique to the function of steroid receptors, such as dissociation from cytoplasmic proteins, nuclear compartmentalization, or ligand-dependent DNA binding. The exact mechanism of calreticulin inhibition awaits further study.

In contrast to our findings with PPAR and RXR, Dedhar et al. (1994) have postulated that calreticulin directly inhibits the function *in vivo* of the nuclear hormone receptor RAR. Thus, in a P19 embryonic carcinoma cell line selected for calreticulin overexpression, retinoic acid-induced expression of specific differentiation-associated markers was suppressed, whereas in a cell line in which calreticulin expression was inhibited by anti-sense RNA, retinoic acid-induced expression of these markers was elevated (Dedhar et al., 1994). It is possible that the observed effects were due to an unusual intracellular trafficking of calreticulin in P19 cells that allows for more efficient nuclear accumulation. However, there was no evidence in this system that calreticulin was modulating the transactivating function or DNA binding capacity of RAR *in vivo* by directly binding to RAR. Since calreticulin is thought to play a multifunctional role in the cell, large changes in the cellular levels of calreticulin may have pleiotropic and indirect effects on gene expression patterns.

In summary, we have shown that calreticulin inhibits the DNA binding of both steroid

and nuclear hormone receptors to their response elements *in vitro*. We have also shown that calreticulin does not have a general role in the *in vivo* regulation of all nuclear hormone receptors.

CHAPTER 4

Interplay of the Peroxisome Proliferator-Activated Receptor and Thyroid Hormone Receptor Signalling Pathways in Regulating Peroxisome Proliferator-Responsive Genes[†]

[†] A version of this chapter has been published. Winrow, C.J., Kassam, A., Miyata, K.S., Marcus, S.M., Hunter, J., Capone, J.P. and Rachubinski, R.A. 1996. Ann. N.Y. Acad. Sci. **804**:214-230. Used with permission from The Annals of the New York Academy of Sciences.

4.1 INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. PPARs are ligand-modulated transcription factors that are activated by structurally diverse xenobiotic chemicals called peroxisome proliferators, as well as by naturally occurring and synthetic fatty acids (Green and Wahli, 1994; Issemann et al., 1993). PPARs modulate transcription of a wide spectrum of genes involved in lipid and metabolic homeostasis, including those encoding the peroxisomal ß-oxidation enzymes fatty acyl-CoA oxidase (AOx) and 3-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (Marcus et al., 1993; Tugwood et al., 1992), mitochondrial medium-chain acyl-CoA dehydrogenase (Gulick et al., 1994), malic enzyme (Castelein et al., 1994), phosphoenolpyruvate carboxykinase (Tontonoz et al., 1995), 3-hydroxy-3-methylglutaryl-CoA synthase (Rodriguez et al., 1994), fatty acid binding protein (Kaikaus et al., 1993), cytochrome P4504A6 (Muerhoff et al., 1992), and apolipoprotein AI (Vu-Dac et al., 1994). PPARs exist in a variety of types, some of which are expressed in cell-, tissue-, and developmentally specific manners and respond differentially to activators. This implies that PPARs play distinct roles in the cell. One example is the demonstration that adipocyte-specific PPARγ2 is a central determinant in regulating the process of adipogenesis (Tontonoz et al., 1994c). PPARs therefore perform essential and diverse functions both in controlling lipid metabolic cascades and in modulating cell differentiation and proliferation.

Regulation of gene expression by PPARs involves the binding of PPARs to specific peroxisome proliferator-response elements (PPREs) through heterodimerization with the 9-*cis* retinoic acid receptor RXRα. Therefore, the retinoid and peroxisome proliferator signalling

pathways converge through the direct interaction of their respective nuclear hormone receptors (Gearing et al., 1993; Issemann et al., 1993; Keller et al., 1993; Kliewer et al., 1992a; Miyata et al., 1994; Tugwood et al., 1992). Moreover, the orphan nuclear hormone receptors COUP-TF1 and HNF-4 bind to specific PPREs and differentially modulate PPAR function (Miyata et al., 1993; Winrow et al., 1994). Gene regulation by PPARs thus appears to be an overall transcriptional response achieved through a dynamic balance among several nuclear hormone receptors functioning as both positive and negative regulators of PPAR activity.

Thyroid hormone receptors (TRs) might be relevant to PPAR function under various physiological conditions. TRs mediate cellular responses to thyroid hormone which, like peroxisome proliferators, affects the expression of many genes important for lipid metabolism, cellular growth, and development. Peroxisome proliferators and thyroid hormones have overlapping metabolic effects, and there is apparent cross-talk between the thyroid hormone and peroxisome proliferator signalling pathways, as thyroid hormone has been shown to attenuate peroxisome proliferator-mediated transcriptional induction of genes encoding peroxisomal ß-oxidation enzymes (Pacot et al., 1993; Takeda et al., 1992). Moreover, a connection between thyroid hormones and peroxisome proliferators is suggested by the finding that PPAR activators such as the fibrate hypolipidemic drugs promote adipose differentiation via a process modulated by thyroid hormone (Gharbi-Chihi et al., 1993).

Recent studies provide evidence that peroxisome proliferator and thyroid hormone signalling pathways converge at the level of their respective nuclear hormone receptors (Bogazzi et al., 1994; Jow and Mukherjee, 1995; Meier-Heusler et al., 1995). Thus, rat PPARα has been shown to negatively regulate the expression of certain genes classically

considered to be thyroid hormone-dependent, either by heterodimerizing directly with particular TR subtypes to generate non-DNA-binding PPAR/TR heterodimers or by forming DNA-binding heterodimers that interact with specific thyroid hormone response elements (TREs). PPARs can also indirectly inhibit TR function by competing with TR for the common auxiliary co-regulator RXR. The converse effects of TR expression on PPAR-mediated activation of peroxisome proliferator-responsive genes have not been addressed. We have examined the effect of rat TRα on the transcriptional activation and DNA-binding properties of rat PPARα (hereafter called TRα and PPAR, respectively). We demonstrate that TRα, on its own as well as cooperatively with RXRα, modulates the transactivation function of PPAR *in vivo* and binds to the AOx-PPRE *in vitro*. Our findings indicate that PPAR and TR signalling pathways converge in the regulation of peroxisome proliferator-responsive genes.

4.2 MATERIALS AND METHODS

4.2.1 Plasmids

The reporter plasmids pCPS*luc* and pAOx(X2)*luc* and the *in vivo* and *in vitro* expression plasmids for rat PPAR and human RXRα have been described in Chapter 2 (Marcus et al., 1993; Miyata et al., 1993; Zhang et al., 1993; Zhang et al., 1992). The mammalian expression vector for rat TRα (pRSV-TRα) was obtained from V. Nikodem (National Institutes of Health, Bethesda) and contains the rTRα cDNA inserted as an *Hind*III/*Hpa*I fragment into pRSV (Bogazzi et al., 1994). The reporter plasmid pTREpal*luc*,

containing a high-affinity palindromic TRE, was obtained from C. Glass (University of California, San Diego)(Glass et al., 1988). The TRα *in vitro* transcription vector was constructed by excising the full-length TRα cDNA from pRSV-TRα and cloning it into pGEM-7Zf(+).

4.2.2. *In Vitro Transcription/Translation*

Transcription/translation was performed using a coupled TNT rabbit reticulocyte lysate system (Promega). Labelled translation products were analyzed on 15% SDS-polyacrylamide gels. Synthesis of proteins for use in gel retardation analysis was carried out in parallel with unlabeled methionine.

4.2.3 Gel Retardation Analysis

Gel retardation analysis was carried out with *in vitro* translated proteins (Miyata et al., 1993). Oligonucleotides corresponding to the AOx-PPRE (5'-CCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT) and the DR4 element 5'-GATCTTCTGACCTCCTGTGACCTGG were annealed to their respective complementary strands and end-labelled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The underlined sequences highlight TGACCT-like direct repeats. One to 2 µl of programmed lysate was incubated for 30 min at 30°C with 0.2 pmol of labelled probe in a final reaction volume of 15 µl containing 7 mM HEPES (pH 7.9), 120 mM NaCl, 1 mM EDTA, 7% (vol/vol) glycerol, 4 µg bovine serum albumin, 8 µg of nonspecific competitor DNA (a 1:1 mixture of poly(dI-dC)·poly(dI-dC) and sonicated salmon sperm DNA), 150 µM

phenylmethylsulfonyl fluoride, and 0.2 mM dithiothreitol. The total amount of lysate in each reaction was kept constant with the addition of unprogrammed lysate. For competition assays, unlabeled annealed oligonucleotides were added to the binding reactions along with labelled probe. For supershift analysis, 1 µl of monoclonal antibodies specific for TRα or polyclonal anti-RXRα antibodies was added to the binding reactions prior to the addition of labelled probe. Protein/DNA complexes were analyzed by electrophoresis at 4°C on prerun 4% polyacrylamide gels (30:1 acrylamide:*N',N'*-methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer.

4.2.4 Transfection and Luciferase Assay

All transfections were carried out with BSC40 monolayer cells by the calcium phosphate method (Miyata et al., 1993). BSC40 cells were chosen for transfection since they contain low endogenous PPARα and RXRα activity. Cells were maintained in medium without phenol red and containing 5% charcoal-stripped fetal bovine serum for 24 h prior to and during transfection. Transfections (using 10 cm dishes of subconfluent cells) contained 5 µg of reporter gene (pCPS*luc*, pAOx(X2)*luc*, or pTREpal*luc*), 0.5 µg to 4 µg of various combinations of receptor expression plasmids (as indicated in the figure legends) and 2 µg of pCH110 (Pharmacia), a ß-galactosidase expression plasmid used as an internal reference. Effector plasmid dosage was kept constant by addition of appropriate amounts of the corresponding empty vectors. Total DNA was normalized to 20 µg by the addition of salmon sperm DNA. Where indicated, the peroxisome proliferator Wy-14,643 or the TR ligand 3,3',5-triiodothyronine (T3; Sigma) was added to final concentrations of 0.1 mM and 10^{-7} M,

respectively (from 100X stocks in dimethyl sulfoxide). Control cells received the equivalent volume of dimethyl sulfoxide. Cell extracts were prepared 48 h after transfection, and luciferase activity was measured. Unless indicated otherwise, values represent the average from at least 3 independent experiments carried out in duplicate and normalized to the internal ß-galactosidase transfection control.

4.3 RESULTS

4.3.1 TRα and TRα/RXRα Heterodimers Bind to the AOx-PPRE

TRs and PPARs bind to cognate response elements composed of direct repeats of the core consensus half site TGACCT, preferentially through heterodimerization with RXR (Tsai and O'Malley, 1994). TRα/RXRα heterodimers bind with high affinity to TGACCT direct repeats spaced by 4 nucleotides (referred to as DR4), whereas PPAR/RXRα heterodimers bind preferentially to direct repeat response elements configured as DR1, as found in both the AOx- and hydratase-dehydrogenase PPREs (Tugwood et al., 1992; Zhang et al., 1993). However, natural TREs are configured in various manners and orientations and can be recognized by TR monomers, homodimers, or heterodimers with RXR and other partners (Desvergne, 1994). To explore whether TRα recognizes the AOx-PPRE and/or modulates PPAR/RXRα protein/DNA interactions on this element, we carried out DNA binding assays with *in vitro* transcribed and translated receptors. TRα/RXRα heterodimers could bind cooperatively to a synthetic DR4 element (Fig. 4-1, compare lanes b and c to lane d). TRα could also bind to the DR4 element weakly as a monomer (Fig. 4-1, lane b). PPAR alone or

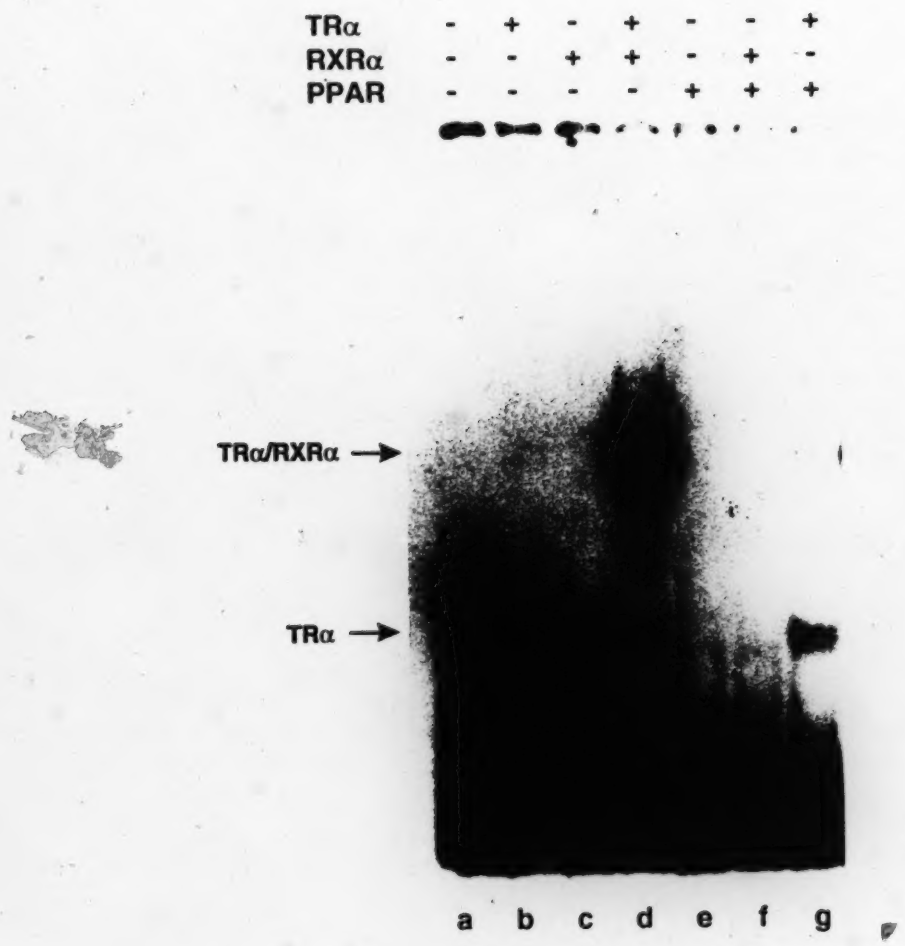


Figure 4-1. Interaction of TRα with a synthetic DR4 element. *In vitro* synthesized TRα, PPARα, and RXRα (1 μl each) were used individually or in pairwise combinations (as indicated at top) in gel retardation assays with a radiolabeled synthetic DR4 probe. Reactions were normalized with unprogrammed lysate as appropriate. The positions of the TRα monomeric complex and the TRα/RXRα heterodimeric complex are indicated. Lane a is a control in which the DR4 probe was incubated with unprogrammed lysate.

with RXRα or TRα did not form a complex on the DR4 element (Fig. 4-1, lanes e, f, and g, respectively).

Mobility shift experiments carried out with the AOx-PPRE are shown in Fig. 4-2. As previously demonstrated (Marcus et al., 1993), PPAR and RXRα did not bind to the AOx-PPRE individually but did bind cooperatively (Fig. 4-2, compare lanes b and d with lane e). Interestingly, TRα alone formed a complex with the AOx-PPRE (Fig. 4-2, lane c). The mobility of this complex was consistent with TRα binding to the AOx-PPRE as a monomer. Additional complexes were not generated when TRα and PPAR were coincubated, indicating that PPAR did not heterodimerize with TRα to bind to the AOx-PPRE (Fig. 4-2, lane g). However, in the presence of RXRα, TRα formed an additional complex on the AOx-PPRE that had a slightly faster electrophoretic mobility than that of the PPAR/RXRα complex (Fig. 4-2, lane f). The TRα/RXRα-dependent complex was generated at an approximately 5-fold lower efficiency *vis-à-vis* the PPAR/RXRα complex when equivalent amounts of the respective receptors were used in the binding assays (Fig. 4-2, compare lane f to lane e). Coincubation of equal amounts of PPAR, RXRα and TRα led to the formation of three distinct complexes, corresponding to PPAR/RXRα heterodimer, TRα/RXRα heterodimer, and TRα monomer complexes (lane h). Under these conditions, the TRα/RXRα and PPAR/RXRα complexes formed with approximately the same efficiency, indicating that TRα and PPAR compete for limiting amounts of RXRα (see below).

To determine if TRα was a component of these protein/DNA complexes, supershift analysis with antibodies to TRα and RXRα were carried out. Addition of anti-TRα antibodies to the binding reaction disrupted the formation of both the faster migrating TRα-dependent

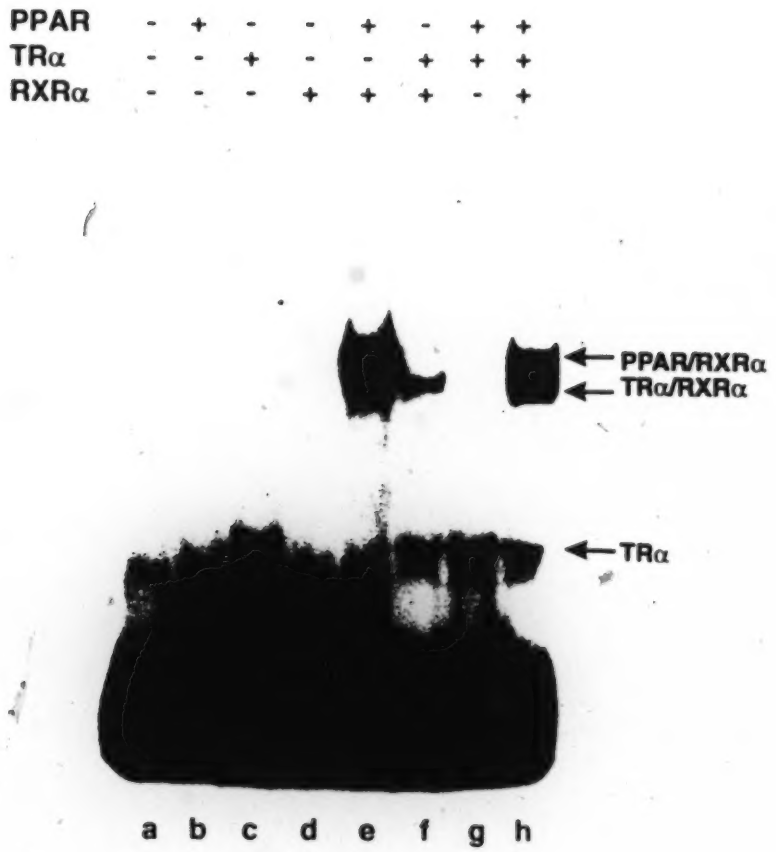


Figure 4-2. TRα binds to the AOx-PPRE on its own and in association with RXRα. *In vitro* synthesized receptors (1 µl each) were incubated individually or in various combinations (as indicated at top) with radiolabeled AOx-PPRE probe and analyzed by gel retardation as in Fig. 4-1. Reactions were normalized with unprogrammed lysate as appropriate. The positions of the monomeric TRα complex and the heterodimeric TRα/RXRα and PPARα/RXRα complexes are indicated. The TRα/RXRα complex has a slightly faster electrophoretic mobility than that of the PPARα/RXRα complex.

complex and the more slowly migrating TRα/RXRα-dependent complex (Fig. 4-3, compare lane c to lane d, and lane e to lane h). In contrast, anti-RXRα antibodies disrupted the formation of only the more slowly migrating complex (Fig. 4-3, compare lane e to lane g). Preimmune serum had no effect on complex formation (Fig. 4-3, lane f). These results show that TRα was present in both the faster migrating TRα-dependent complex and the more slowly migrating TRα/RXRα-dependent complex, whereas RXRα was only a component of the more slowly migrating complex.

Addition of the TR ligand T3 to a binding reaction containing TRα and RXRα resulted in a slightly increased mobility of the TRα/RXRα complex but had little effect on its intensity (Fig. 4-3, compare lane j to lane i). This altered mobility may be related to previous observations which demonstrated that T3 induces a ligand-dependent conformational change in TR/RXR heterodimers (Leng et al., 1993).

The TRα/RXRα-dependent complex was sequence-specific, since it could be competed with unlabeled AOx-PPRE probe DNA but not with a nonspecific competitor oligonucleotide (Fig. 4-4; compare lane d to lanes e-g and to lanes i-k, respectively). The TRα monomeric complex could also be competed with specific, but not with nonspecific, competitor DNA; however, this competition required at least 100- fold molar excess of competitor DNA compared to the 10- to 50-fold molar excess that was sufficient to disrupt the PPAR/RXRα and TRα/RXRα complexes. These results suggest that the TRα monomeric complex is more stable than the TRα/RXRα complex. Our findings demonstrate that the TRα binds in a sequence-specific manner to the AOx-PPRE on its own and as a heterodimer with RXRα.

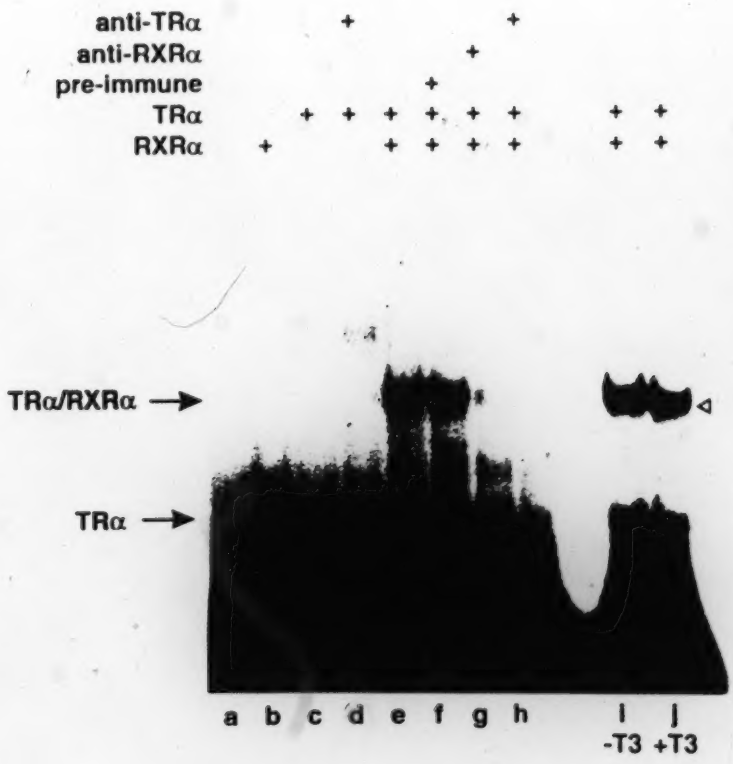


Figure 4-3. TRα is present in complexes formed on the AOx-PPRE. Gel retardation assays using radiolabeled AOx-PPRE were carried out with *in vitro* synthesized receptors (1 μl each) in the presence of preimmune serum (lane f), monoclonal anti-TRα antibodies (lanes d and h), or polyclonal anti-RXRα antibodies (lane g), as indicated. 1 μl of preimmune serum or antibodies was added along with the indicated receptors prior to the addition of the AOx-PPRE probe. Reactions were normalized with unprogrammed lysate as appropriate. Inclusion of anti-TRα antibodies resulted in the loss of both the fast migrating and slowly migrating complexes (lanes d and h), while inclusion of anti-RXRα antibodies resulted in the loss of only the slowly migrating complex (lane g). Specific supershifted complexes produced by addition of anti-TRα and anti-RXRα antibodies were apparent after longer exposure. Lane a is a control in which the AOx-PPRE probe was incubated with unprogrammed lysate. Lanes i and j are identical in composition to lane e, except that lane j contained T3 (10^{-7} M final concentration from a stock in dimethyl sulfoxide), while lane i contained an equal volume of dimethyl sulfoxide alone. T3 led to a slight and reproducible increase in the electrophoretic mobility of the TRα/RXRα complex, as indicated by the arrowhead.

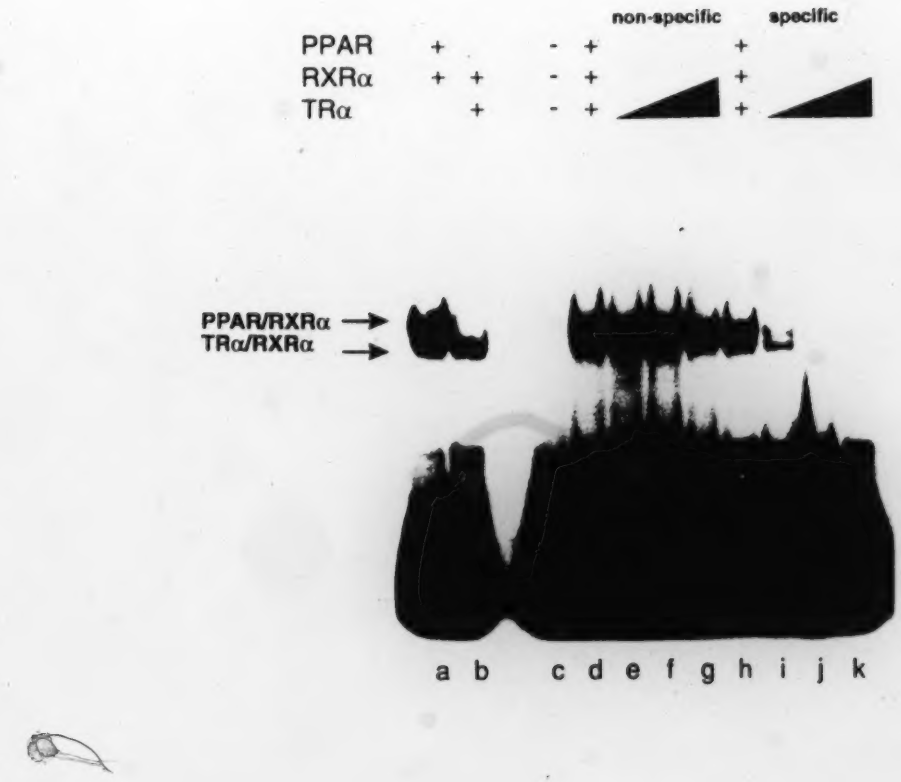


Figure 4-4. TRα binds to the AOx-PPRE in a sequence-specific manner. *In vitro* synthesized RXRα, TRα, and PPARα (1 µl each) were coincubated with radiolabeled AOx-PPRE alone (lanes d and h) and in the presence of 10-, 50- and 100-molar excess of unlabeled, double-stranded nonspecific competitor DNA (5'GATCCCTACCCATACGACGTCCCAGACTACGCTTGAGCT and its complement; lanes e-g, respectively) or 10-, 50- and 100-molar excess of unlabeled AOx-PPRE (lanes i-k, respectively) and analyzed by gel retardation. Lanes a and b are incubations carried out with combinations of *in vitro* translated PPARα + RXRα and of TRα + RXRα (1 µl of each), respectively, to indicate the positions of the heterodimeric complexes. Lane c is a control in which the AOx-PPRE probe was incubated with unprogrammed lysate.

4.3.2 TRα Competes with PPAR for Binding to the AOx-PPRE

As indicated above (Fig.4-2, lane h), coincubation of the AOx-PPRE with equal amounts of TRα, PPAR, and RXRα resulted in the formation of three species of complex corresponding to the TRα monomeric complex, the PPAR/RXRα complex, and the TRα/RXRα complex. When constant amounts of PPAR and RXRα were coincubated with increasing amounts of TRα, the PPAR/RXRα complex gradually disappeared concomitantly with an increase in the intensity of the TRα/RXRα complex and the TRα monomeric complex (Fig. 4-5, lanes b-d). This result could be due to a competition of TRα for limiting amounts of RXRα, a competition of TRα/RXRα heterodimers for the AOx-PPRE binding site, and/or the formation of non-DNA-binding TRα/PPAR heterodimers. These possibilities are addressed in Fig. 4-6 in which binding reactions were carried out with increasing amounts of RXRα and/or PPAR while keeping the amount of TRα constant. Increasing the RXRα concentration led to an increase in the amount of TRα/RXRα complex concomitantly with a decrease in the amount of TRα monomeric complex. This result would suggest that RXRα was limiting for complex formation under these conditions (Fig. 4-6, compare lane a to lanes b and c). Incubation of TRα with increasing PPAR led to a decrease in the formation of the TRα monomeric complex, suggesting that PPAR can sequester TRα (Fig. 4-6, compare lane a to lanes d and e). Coincubation of TRα and PPAR with increasing amounts of RXRα led to a reduction in the amount of TRα monomeric complex concomitant with increased amounts of the PPAR/RXRα and TRα/RXRα complexes (Fig. 4-6, compare lane a to lanes f and g). These results suggest that TRα can inhibit binding of PPAR to the AOx-PPRE by competing for the common heterodimerization partner RXRα

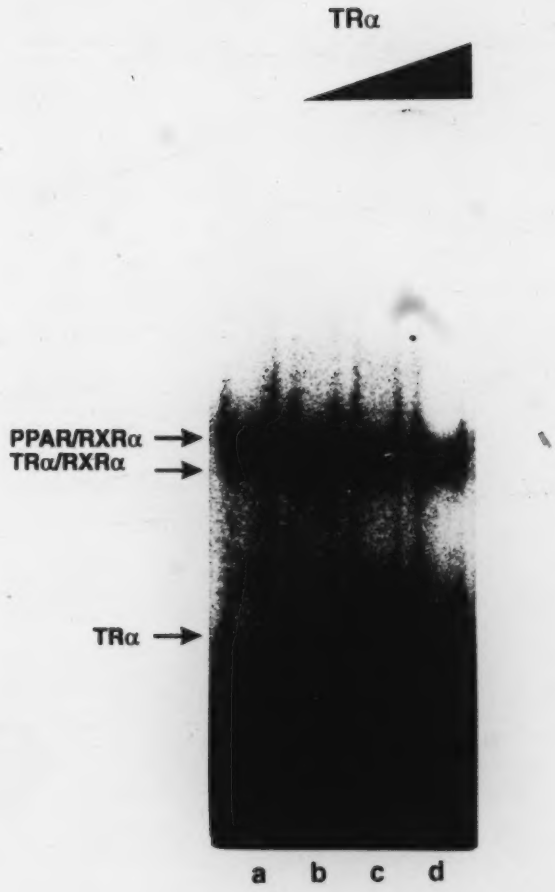


Figure 4-5. TRα competes with PPARα/RXRα for binding to the AOx-PPRE. *In vitro* translated PPARα and RXRα (2 µl of each) were incubated in the absence (lane a) or presence of 2, 4, and 6 µl of *in vitro* translated TRα (lanes b, c and d, respectively) and analyzed by gel retardation. Volumes of lysate were kept constant by the addition of unprogrammed lysate as appropriate. TRα inhibits the formation of the PPARα/RXRα complex concomitant with the formation of the TRα/RXRα complex and the TRα complex.

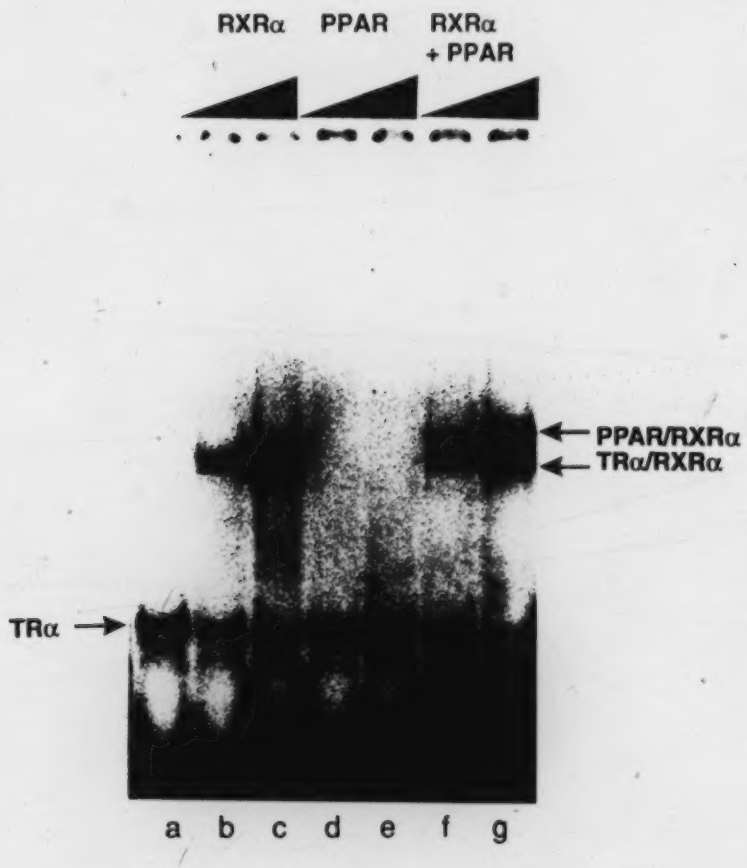


Figure 4-6. Effects of increasing amounts of RXRα and PPARα on the formation of TRα-dependent protein/DNA complexes. *In vitro* translated TRα (2 µl) was mixed with RXRα (2 µl, lane b; 5 µl, lane c), PPARα (2 µl, lane d; 5 µl, lane e), PPARα (1 µl) + RXRα (1 µl) (lane f) or PPARα (1 µl) + RXRα (4 µl) (lane g) in the presence of radiolabeled AOx-PPRE and analyzed by gel retardation. Lysate volumes were kept constant by the addition of unprogrammed lysate as appropriate.

and by binding to the AOx-PPRE on its own or cooperatively with RXRα. Moreover, the availability of PPAR/RXRα heterodimers capable of binding to the AOx-PPRE may also be restricted through the formation of non-PPRE-binding TRα/PPAR complexes.

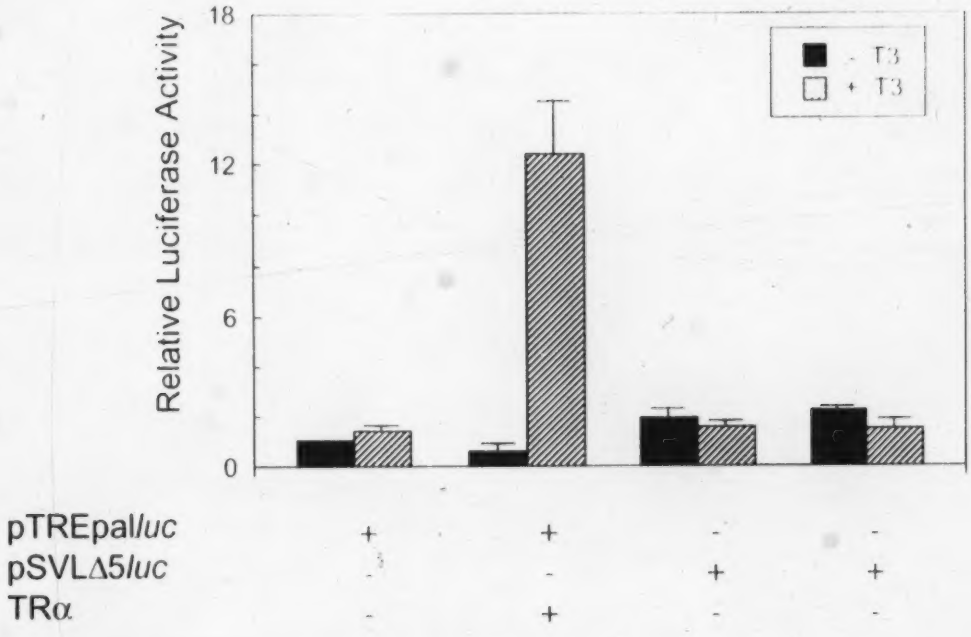
4.3.3 Unliganded TRα Stimulates Transactivation by PPAR/RXRα Heterodimers

Transient transfection assays were carried out in BSC40 cells to examine the effects of expression of TRα on PPAR/RXRα-dependent transactivation of a reporter gene plasmid containing two copies of the AOx-PPRE (pAOx(X2)*luc*). Control transfections showed the T3-dependent activation of a luciferase reporter gene plasmid containing a palindromic TRE (pTREpal*luc*) following cotransfection with the TRα expression vector (Fig. 4-7A). TRα functions as a ligand-dependent transactivator in BSC40 cells, resulting in a 10- to 12-fold T3-dependent induction of the TRE reporter gene construct. Expression from the parental reporter gene plasmid not containing a TRE (pSVLΔ5'*luc*) was not affected by expression of TRα or by the presence of T3, as reported previously (Glass et al., 1988).

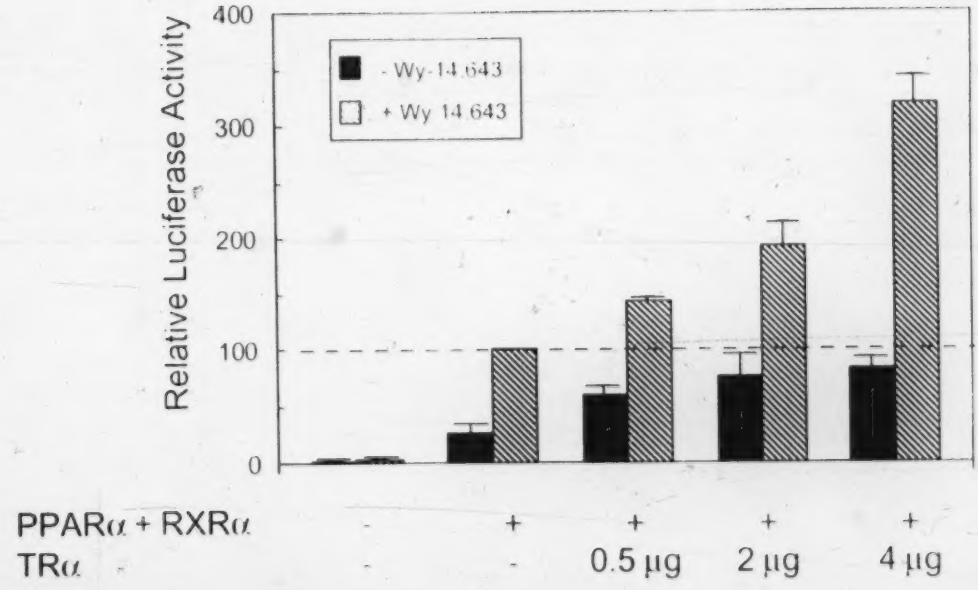
Fig. 4-7B shows the effects of transfecting increasing amounts of the TRα expression plasmid on transactivation of the AOx-PPRE reporter gene plasmid by PPAR and RXRα. Cotransfection of PPAR and RXRα expression plasmids resulted in an approximately 7- to 10-fold activation of expression of the AOx-PPRE reporter gene plasmid over basal levels in the absence of exogenously added PPAR activators, as previously demonstrated (Marcus et al., 1993). This activator-independent induction is presumably due to the presence of endogenous PPAR activators. Addition of the potent peroxisome proliferator Wy-14,643 to the transfections resulted in a 50-fold stimulation of activity of the AOx-PPRE reporter

Figure 4-7. TRα enhances transactivation by PPARα/RXRα *in vivo*. (*A*) BSC40 cells were cotransfected with the reporter plasmids pTREpal*luc* or pSVLΔ5'*luc* and the TRα expression plasmid pRSV-TRα in the presence or absence of T3, as indicated. Luciferase assays were performed as indicated in **Materials and Methods**. The values reported are the averages (± SEM) of 3 independent transfections carried out in duplicate and normalized to the value obtained with pTREpal*luc* alone (taken as 1). (*B*) BSC40 cells were transfected with pAOx(X2)*luc* in the presence or absence of the peroxisome proliferator Wy-14,643, along with a constant amount of PPARα and RXRα expression plasmids (2 μg each) and increasing amounts of the TRα expression plasmid (in μg), as indicated. Plasmid dosage was normalized in each case with appropriate amounts of the corresponding empty expression vectors. The values reported are the averages (± SEM) of a minimum of 3 independent transfections carried out in duplicate and normalized to the value obtained from Wy-14,643-treated cells cotransfected with PPARα and RXRα expression plasmids (taken as 100%). The results show that TRα potentiates Wy-14,643-mediated transactivation by PPARα/RXRα.

A


Relative Luciferase Activity
18
12
6
0
- T3
+ T3
pTREpal*luc*
pSVLΔ5*luc*
TRα

B


Relative Luciferase Activity
400
300
200
100
0
- Wy-14,643
+ Wy 14,643
PPARα + RXRα
TRα
0.5 μg
2 μg
4 μg

gene plasmid over basal levels. Surprisingly, cotransfection with increasing amounts of the TRα expression plasmid resulted in progressively enhanced stimulation by PPAR/RXRα. Cotransfection of up to 4 μg of the TRα expression plasmid (representing a 2-fold molar excess over PPAR/RXRα) resulted in a 100- to 200-fold stimulation of Wy-14,643-dependent, PPAR/RXRα-mediated transactivation of the AOx-PPRE reporter gene plasmid over basal levels. TRα also stimulated the Wy-14,643-independent induction by PPAR/RXRα, although to a lesser extent (40- to 50-fold stimulation over basal levels compared to 10- to 20-fold stimulation in the absence of TRα).

Transfection of the PPAR expression vector alone resulted in a modest (10-fold) stimulation of AOx-PPRE reporter gene activity in the presence of Wy-14,643, likely because of interaction between PPAR and endogenous RXRα and/or other cofactors (Fig. 4-8). However, TRα did not further stimulate PPAR-mediated activation under these conditions. Indeed, activation was reduced by approximately 50% when a 2-fold excess of TRα expression plasmid was transfected. Similarly, the slight activation of AOx-PPRE reporter gene expression mediated by RXRα alone (2- to 4-fold) was not appreciably affected by cotransfection of the TRα expression vector. Interestingly, TRα on its own stimulated reporter gene expression approximately 10-fold, but only when transfected at relatively high amounts. However, this TRα-mediated induction was not further stimulated by Wy-14,643, as compared to control transfections.

To examine the requirements of a PPRE for TRα-dependent stimulation, cotransfection experiments similar to those reported above were carried out with pCPS*luc*, the parental vector used to construct pAOx(X2)*luc*. Transfection of PPAR/RXRα alone, or

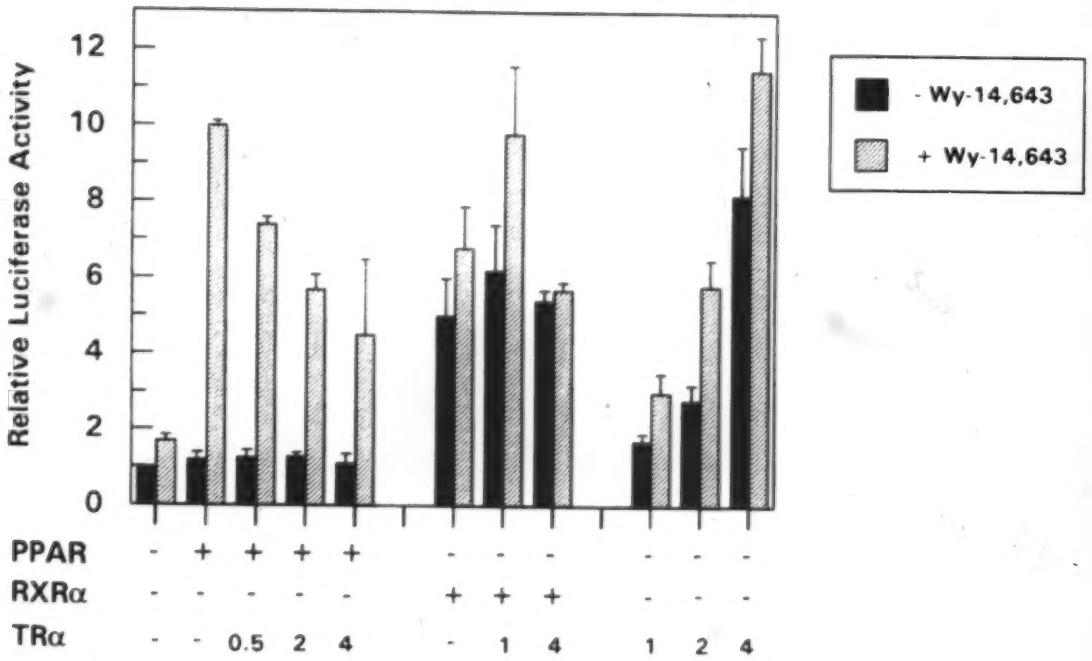


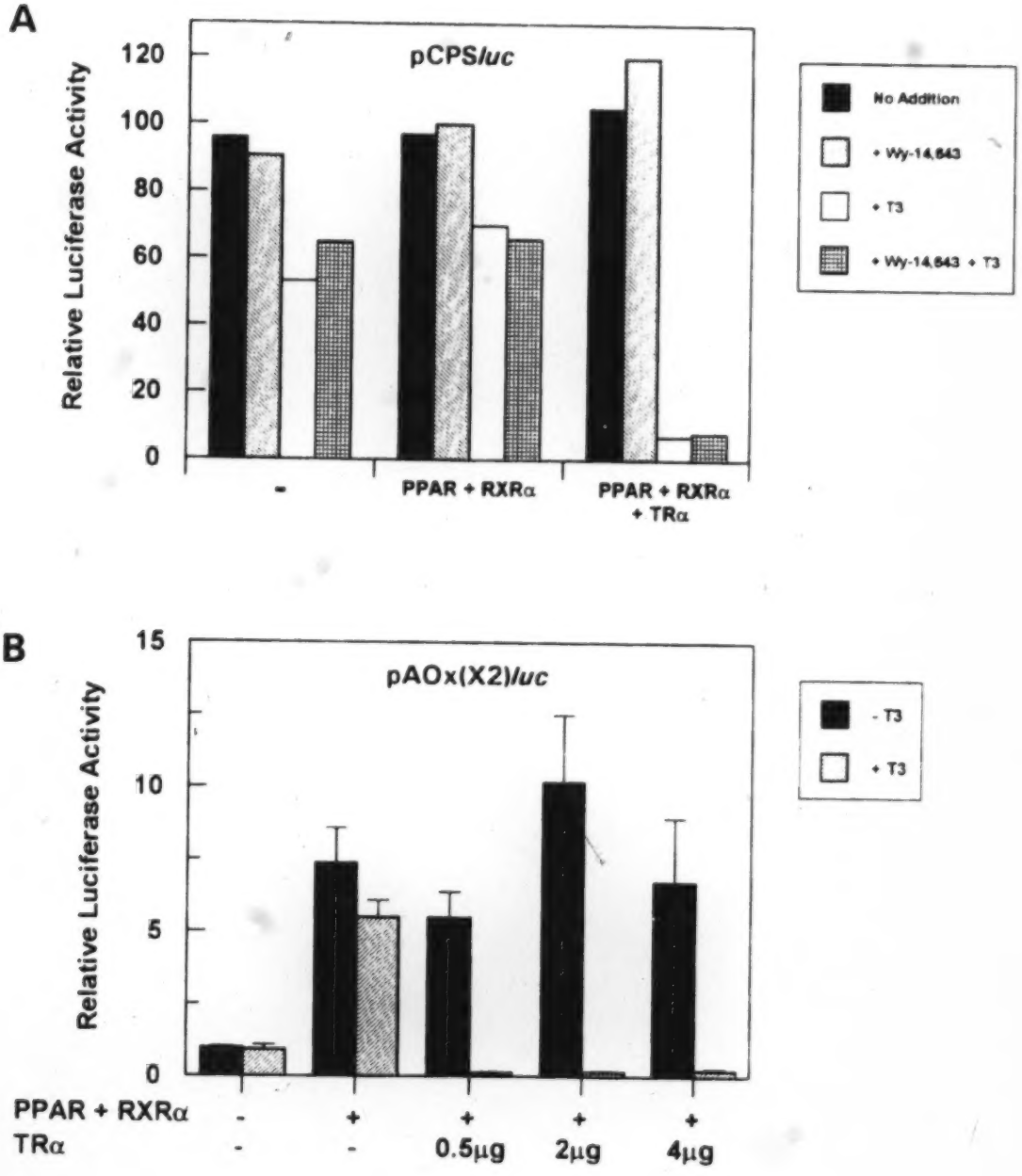
Figure 4-8. TRα-mediated stimulation of Wy-14,643-dependent activation requires the presence of PPARα and RXRα. The TRα expression plasmid pRSV-TRα (in μg) was cotransfected with pAOx(X2)*luc* alone or in combination with plasmids expressing PPARα (2 μg) or RXRα (2 μg) in the presence or absence of Wy-14,643, as indicated. The values reported are the averages (± SEM) of three independent transfections carried out in duplicate and normalized to the value obtained for cells transfected with the pAOx(X2)*luc* reporter plasmid alone (taken as 1).

in combination with TRα, had little effect on the expression of this reporter gene construct, either in the absence or presence of Wy-14,643 (Fig. 4-9A). Therefore, TRα-mediated stimulation of Wy-14,643-dependent transcriptional induction requires both exogenously added PPAR and RXRα and a functional PPRE.

In contrast to the stimulation of transactivation of the AOx-PPRE by PPAR/RXRα observed with unliganded TRα, addition of T3 to the transfections resulted in an almost complete repression of both Wy-14,643-independent transcriptional induction (Fig. 4-9B) and Wy-14,643-dependent transcriptional induction (Fig. 4-10) Repression was manifested through TRα, since T3 alone had no effect on basal level expression of the AOx-PPRE reporter gene plasmid or on transactivation by PPAR/RXRα (Fig. 4-9B). However, T3-mediated, TRα-dependent repression was independent of PPAR/RXRα or on the presence of a PPRE, since inhibition of basal level activity was also observed with the parental reporter plasmid pCPS*luc* (Fig. 4-9A). These results suggest that liganded TRα has a generalized repressive effect on transcription, perhaps because it sequesters common co-activators required for basal level and/or activated transcription of the minimal promoters used (see **DISCUSSION**). This would be consistent with the observation that liganded TRα reduced activity below the basal levels obtained from cells transfected with pAOx(X2)*luc* or pCPS*luc* alone.

Overall, our findings suggest that TRα in the unliganded state is a positive modulator of PPAR/RXRα-mediated transactivation of the AOx-PPRE, despite the fact that *in vitro*, TRα appears to inhibit the binding of PPAR/RXRα heterodimers to this response element.

Figure 4-9. TRα inhibits transactivation in the presence of T3. (*A*) BSC40 cells were cotransfected with the reporter gene pCPS*luc* and plasmids (2 μg each) expressing the indicated receptors in the presence or absence of Wy-14,643 and T3, as indicated. The values reported are the averages of duplicate transfections and are normalized to the value obtained with pCPS*luc* cotransfected with PPARα and RXRα expression plasmids in the presence of Wy-14,643 (taken as 100%). Readings of duplicate samples did not vary by more than 15%. (*B*) BSC40 cells were cotransfected with pAOx(X2)*luc*, PPARα and RXRα expression plasmids (2 μg each), and increasing amounts of the TRα expression plasmid pRSV-TRα in the presence or absence of T3, as indicated. The values reported are the averages (± SEM) from 3 independent transfections carried out in duplicate and are normalized to the value obtained for cells transfected with the reporter gene pAOx(X2)*luc* alone (taken as 1).


A
pCPS/luc
Relative Luciferase Activity
120
100
80
60
40
20
0
-
PPAR + RXRα
PPAR + RXRα + TRα
No Addition
+ Wy-14,643
+ T3
+ Wy-14,643 + T3
B
pAOx(X2)/luc
Relative Luciferase Activity
15
10
5
0
- T3
+ T3
PPAR + RXRα - + + + +
TRα - - 0.5μg 2μg 4μg

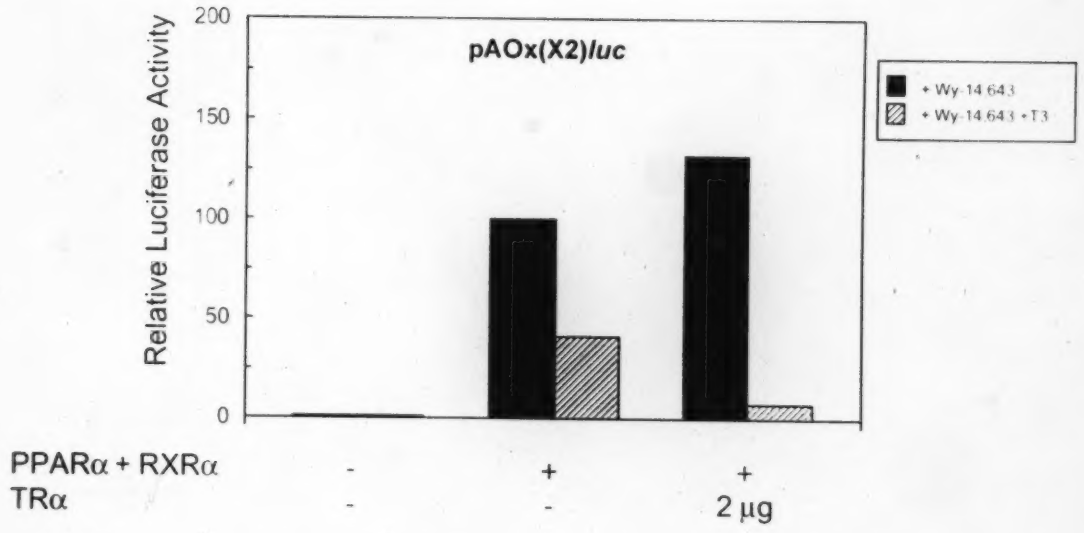


Figure 4-10. Liganded TRα also inhibits Wy-14,643-mediated transactivation by PPARα/RXRα. Transient co-transfections were performed with 5 µg of pAOx(X2)*luc*, 2 µg each of PPARα and RXRα, and 2 µg of TRα expression plasmids, as indicated. Transfection media was supplemented with Wy-14,643 alone or with both Wy-14,643 and T3, as shown in the figure legend. The values presented represent the average of duplicate transfections, and are normalized to the value obtained with PPARα/RXRα in the presence of Wy-14,643 (taken as 100%).

4.4 DISCUSSION

Recent findings from several groups have indicated that PPAR can differentially influence TR-mediated activation of thyroid-hormone responsive genes (Bogazzi et al., 1994; Jow and Mukherjee, 1995; Meier-Heusler et al., 1995). Conversely, our findings show that TRα modulates PPAR-mediated transactivation of peroxisome proliferator-responsive genes *in vivo* and can disrupt the binding of PPAR/RXRα heterodimers to PPREs. Thus, cross-talk and coupling between the thyroid hormone and peroxisome proliferator signalling pathways are important to the reciprocal regulation of both thyroid hormone- and peroxisome proliferator-responsive genes.

We have shown that TRα is capable of binding to the AOx-PPRE in a sequence-specific manner on its own as well as through association with RXRα. This is not necessarily unexpected, since thyroid receptors bind promiscuously as monomers or as homo- and heterodimers to a wide variety of structurally diverse response elements configured in different manners (Desvergne, 1994). Therefore, the AOx-PPRE defines a novel target for TRα. This finding, coupled with our previous observations that COUP-TF1 (Miyata et al., 1993) and HNF-4 (Chapter 2) also bind to the AOx-PPRE, provides further evidence that the AOx-PPRE is a composite response element that is a target for multiple members of the nuclear hormone receptor superfamily.

The binding of TRα and of TRα/RXRα to the AOx-PPRE led to a decrease in the binding of PPAR/RXRα to this element. Our findings suggest that this decrease was the net result of TRα competing for the common heterodimerization partner RXRα and for binding to the AOx-PPRE. Moreover, as shown by others, PPAR and TRα can heterodimerize in

solution, indicating that TRα can sequester PPAR as well as RXRα (Bogazzi et al., 1994). However, the TRα/PPAR heterodimer was unable to bind to the AOx-PPRE, and a natural DNA binding site for the TRα/PPAR heterodimer pair, if it exists, remains to be identified. Recently, the TRß/PPAR heterodimer has been shown to be capable of binding to a DR2 element present in the promoter region of the gene encoding myelin proteolipid protein (Bogazzi et al., 1994).

Paradoxically, cotransfection of PPAR/RXRα with the TRα expression vector led to a significant Wy-14,643-mediated stimulation of transcription of the AOx-PPRE reporter gene over that seen with PPAR/RXRα alone. This stimulation required the presence of both RXRα and PPAR and a functioning PPRE in the reporter gene plasmid. In the absence of exogenously added RXRα, TRα led to a reduction in PPAR/Wy-14,643-dependent stimulation. TRα on its own gave a slight stimulation of AOx-PPRE reporter gene expression; however, this was negated by exogenously added RXRα or PPAR. Neither TRα alone nor in combination with RXRα conferred responsiveness of the AOx-PPRE to Wy-14,643 *in vivo*. These findings suggest that TRα cooperates with RXRα and PPAR to stimulate Wy-14,643-dependent transactivation of the AOx-PPRE.

The modest transcriptional activation of the AOx-PPRE observed with TRα on its own is inconsistent with the reports that activation by TRs is normally hormone dependent. Indeed, unliganded TRs typically repress the basal activity of promoters that contain TREs through active silencing or inhibition of formation of the preinitiation complex (Fondell et al., 1993). Nevertheless, examples of ligand-independent transactivation by TRα have been reported (Forman et al., 1988; Saatcioglu et al., 1993), and therefore it remains possible that

DNA-bound TRα directly contributes to the overall enhanced stimulation by PPAR/RXRα.

The results showing that unliganded TRα augments PPAR/RXRα-mediated transactivation of the AOx-PPRE *in vivo* appear to be at odds with the *in vitro* results showing that TRα inhibits the binding of PPAR/RXRα to the AOx-PPRE. It is possible that TRα may not bind to the AOx-PPRE *in vivo*, perhaps because PPAR/RXRα binds more avidly to the AOx-PPRE or because there exist additional cellular factors that stabilize PPAR/RXRα interactions on the AOx-PPRE. TRα-dependent stimulation of PPAR/RXRα activity *in vivo* may thus be manifested though mechanisms that do not involve the binding of TRα to the AOx-PPRE. For instance, it is possible that TRα titrates, and therefore counteracts, the effects of a repressor that attenuates transcriptional activation by PPAR/RXRα heterodimers, thereby relieving repression. Consistent with this possibility, unliganded TRα has been shown to bind to an inhibitory factor that also interacts with the retinoic acid receptor, RARα (Casanova et al., 1994). Therefore, overexpression of TRα may lead to an alteration in the repertoire of RXR heterodimers present in the cell, which could effectively increase the amount of RXRα available for interaction with PPAR.

Interestingly, the addition of T3 led to strong TRα-dependent inhibition of transcription of the AOx-PPRE both in the presence and absence of Wy-14,643. This finding may be relevant to the fact that administration of thyroid hormone can antagonize activation of the AOx gene by peroxisome proliferators (Yamada et al., 1994). However, transcriptional repression by liganded TRα appeared to be indirect, since repression was also observed in the absence of PPAR/RXRα or with the reporter gene construct pCPS*luc*, which does not contain a PPRE. Transcriptional repression by liganded TRα may be due to its ability, but not that

of unliganded TRα, to squelch transcription by sequestering general auxiliary transcription factors.

Negative transcriptional effects of liganded TRα resulting from squelching do not necessarily exclude liganded TRα from having a more direct negative effect on PPAR/RXRα activation. TRs have complex and diverse effects on transcriptional control, since they can act as both positive and negative regulators of gene expression depending on the particular response element, the heterodimerization partner, and the absence or presence of ligand. TRα/RXRα heterodimers undergo a ligand-dependent conformational change (Bendik and Pfahl, 1995; Leng et al., 1993). It is interesting to note that the addition of T3 led to a slight, but reproducible, altered electrophoretic mobility of the TRα/RXRα complex on the AOx-PPRE, suggesting a ligand-dependent conformational change on this element. Therefore, it remains possible that TRα/RXRα heterodimers bound to the AOx-PPRE *in vivo* can actively repress transcription in the presence of ligand.

In summary, we have established a link between PPARs and TRs that may be physiologically important in the regulation of lipid homeostasis and in mediating the thyromimetic effects of peroxisome proliferators. The mechanisms by which TRα differentially modulates transactivation by PPARs, and *vice versa*, are complex and appear to be dependent upon a dynamic balance between RXRα, PPAR, TRα, and possibly other cellular factors and nuclear hormone receptors. Elucidation of these pathways will provide a clearer understanding of the mechanism underlying the pleiotropic cellular effects mediated by PPARs.

CHAPTER 5

Cross-talk Between Orphan Nuclear Hormone Receptor RZRα and Peroxisome Proliferator-Activated Receptor α in Regulation of the Peroxisomal Hydratase-Dehydrogenase Gene †

† A version of this chapter has been published. 1998. Winrow, C.J., Capone, J.P. and Rachubinski, R.A. J. Biol. Chem. **273**:31442-31448. Used with permission from The American Society for Biochemistry and Molecular Biology.

5.1 ABSTRACT

The genes encoding the peroxisomal β-oxidation enzymes enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) and fatty acyl-CoA oxidase (AOx) are coordinately regulated by peroxisome proliferator-activated receptor α (PPARα)/9-*cis* retinoic acid receptor (RXRα) heterodimers that transactivate these genes in a ligand-dependent manner via upstream peroxisome proliferator-response elements (PPRE). Here we demonstrate that the monomeric orphan nuclear hormone receptor, RZRα, modulates PPARα/RXRα-dependent transactivation in a response-element dependent manner. Electrophoretic mobility shift analysis showed that RZRα bound specifically as a monomer to the HD-PPRE but not the AOx-PPRE. Determinants in the HD-PPRE for binding of RZRα were distinct from those required for interaction with PPARα/RXRα heterodimers. In transient transfections, RZRα stimulated ligand-mediated transactivation by PPARα from an HD-PPRE luciferase reporter in the absence of exogenously added RXRα, but did not affect PPARα-dependent transactivation of an AOx-PPRE reporter gene. These data illustrate cross-talk between the RZRα and PPARα signaling pathways at the level of the HD-PPRE in the regulation of the HD gene and characterize additional factors governing the regulation of peroxisomal β-oxidation.

5.2 INTRODUCTION

Nuclear hormone receptors are a diverse group of structurally related ligand-activated transcription factors that direct the expression of target genes in response to physiological and environmental stimuli (Mangelsdorf and Evans, 1995; Tsai and O'Malley, 1994). Peroxisome proliferator-activated receptors (PPAR) are members of the steroid/nuclear hormone receptor superfamily that act to regulate a large number of genes involved in differentiation and lipid metabolism (Juge-Aubry et al., 1997; Latruffe and Vamecq, 1997; Spiegelman and Flier, 1996; Tontonoz et al., 1995; Tontonoz et al., 1994b; Tugwood et al., 1992; Zhang et al., 1992) in response to a variety of compounds collectively called peroxisome proliferators. Peroxisome proliferators include the fibrate family of hypolipidemic drugs, phthalate ester plasticizers, herbicides, pesticides, antidiabetic thiazolidinediones, as well as natural and synthetic fatty acids (Forman et al., 1997b; Kliewer et al., 1995; Kliewer et al., 1997; Lambe and Tugwood, 1996; Lehmann et al., 1995; Lock et al., 1989). Transactivation of target genes by PPARs is mediated through binding to *cis*-acting regulatory sequences called peroxisome proliferator-response elements (PPRE) that consist of direct repeats of the hexameric TGACCT/C core motif. PPARs heterodimerize with the 9-*cis* retinoic acid receptor, RXRα, and bind with preference to response elements with spacing of one nucleotide between hexameric repeats (DR1) (Chu et al., 1995a; Keller et al., 1993; Kliewer et al., 1992b; Marcus et al., 1993). PPREs have been identified in the regulatory regions of a number of genes, including those encoding the first two enzymes of the peroxisomal β-oxidation pathway: fatty acyl-CoA oxidase (AOx) (Osumi et al., 1991; Tugwood et al., 1992) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) (Bardot et al., 1993;

Zhang et al., 1992).

In addition to PPARs, several other nuclear hormone receptors have been shown to bind to PPREs and differentially modulate PPAR function. These include chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Miyata et al., 1993), hepatocyte nuclear factor-4 (HNF-4) (Chapter 2) and thyroid hormone receptor (TR) (Chapter 4). Transcriptional regulation via PPREs is thus a net aggregate response manifested in part by the availability of PPARs and other factors that bind to PPREs, the complexity of response elements, and the interplay of PPARs with other nuclear hormone receptors and cofactors. Overlying this network is a series of co-repressors and co-activators that serve to mediate receptor signaling. The overall intricacy of the system enables the integration of information from multiple signaling pathways to ensure appropriate transcriptional responses of target genes to various stimuli.

The retinoid Z receptor (RZR) family, also known as the retinoid orphan receptor (ROR) family, is a recently described group of nuclear hormone receptors shown to be involved in regulating genes responsible for cellular differentiation, the inflammatory response, and lipid metabolism (Carlberg et al., 1994; Giguere et al., 1994; Hirose et al., 1994; Vu-Dac et al., 1997). RZR/ROR target response elements have been identified in the promoters of genes for chicken γF-crystallin, human and rat bone sialo protein, human 5-lipoxygenase, N-myc proto-oncogene, and apolipoprotein AI (Dussault and Giguere, 1997; Schrader et al., 1996; Steinhilber et al., 1995; Tini et al., 1995; Vu-Dac et al., 1997). Three RZR/ROR isoforms have been characterized (α, β, γ) that show differential tissue localization. The α isoform includes four splice variants (RORα1, RORα2, RORα3 and RZRα) and is

widely expressed, RZRβ is found primarily in brain tissues, and RORγ is localized to skeletal muscle (Becker-Andre et al., 1993; Carlberg et al., 1994; Hirose et al., 1994; Missbach et al., 1996). While nuclear hormone receptors typically bind response elements as hetero- or homodimers, RZR/ROR family members are distinct in that they are able to bind to, and transactivate from, target response elements as monomers (Carlberg et al., 1994; Giguere et al., 1994). RZR/ROR receptors recognize a TGACCT/C consensus half-site and have a demonstrated preference for sites flanked by a 6 base A/T-rich region (Carlberg et al., 1994; Giguere et al., 1995; Giguere et al., 1994; Schrader et al., 1996). This consensus half site is embedded in the HD-PPRE, and recently RZRα was shown to bind to this element, suggesting that RZRα might be a candidate regulator of peroxisomal β-oxidation (Schrader et al., 1996). To explore this possibility, I undertook to determine how RZRα might interact with PPARα and RXRα in regulating the transcription of genes encoding the first two enzymes of the peroxisomal β-oxidation pathway, AOx and HD. The results presented here indicate that RZRα binds to the HD-PPRE but not the AOx-PPRE, and selectively potentiates transactivation from the HD-PPRE in a manner dependent on the relative availability of RXRα.

5.3 MATERIALS AND METHODS

5.3.1 Plasmids

The *in vitro* and *in vivo* expression plasmids for rat PPARα and human RXRα, and the luciferase reporter constructs pCPS*luc* containing the minimal promoter for the gene

encoding carbamoyl phosphate synthetase, pHD(X3)*luc* containing two copies of the minimal HD-PPRE, and pAOx(X2)*luc* containing two copies of the minimal AOx-PPRE, have been described previously in Chapter 2 (Marcus et al., 1993; Zhang et al., 1993; Zhang et al., 1992). The plasmid pM2(X3)*luc*, constructed by cloning the mutant HD-PPRE synthetic oligonucleotide M2 (5'-GATCCTCTCCTTTAAAATATTGAACTATTAC-CTACATTTGA) and its complement into the *Bam*HI site of pCPS*luc*, contains three direct tandem copies of the M2 element. The expression plasmid for human RZRα (RZRα/SG5) (Schrader et al., 1996) was a kind gift of Carsten Carlberg (Hôpital Cantonal Universitaire de Genève, Geneva, Switzerland).

5.3.2 In Vitro Transcription/Translation

Transcription/translation of cDNAs encoding PPARα, RXRα and RZRα was performed using the TNT T7 coupled rabbit reticulocyte lysate system according to the manufacturer's protocol (Promega). Translation products labeled with *L*-[^{35}S]methionine were analyzed on 15% SDS-polyacrylamide gels. Synthesis of proteins for use in electrophoretic mobility shift analysis (EMSA) was carried out in parallel with unlabeled methionine.

5.3.3 Electrophoretic Mobility Shift Analysis

EMSA was carried out as previously described in Chapter 2 (Miyata et al., 1993; Zhang et al., 1993) with oligonucleotide probes corresponding to the HD-PPRE (5'-CCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA and its complement) and the

AOx-PPRE (5'-CCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT and its complement). Underlined sequences indicate TGACCT-like direct repeats. Complementary oligonucleotides were annealed and end-labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. 1 to 2 µl of programmed lysate was incubated with 0.2 pmol of labeled probe at 25°C in a final volume of 15 µl containing 6 mM Hepes (pH 7.9), 120 mM NaCl, 0.4 mM $MgCl_2$, 0.1 mM EDTA, 7% (v/v) glycerol, 4 µg bovine serum albumin, 4 µg nonspecific competitor DNA (poly dI•dC and sonicated salmon sperm DNA, 1:1 weight ratio), 150 µM phenylmethylsulfonyl fluoride and 0.2 mM dithiothreitol. The total amount of reticulocyte lysate in each reaction was kept constant by addition of unprogrammed lysate. Binding reactions were analyzed by electrophoresis at 4°C on prerun 3.5% polyacrylamide gels (30:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer, followed by autoradiography.

5.3.4 *Transient Transfections and Measurement of Luciferase Activity*

Transfections were performed in African green monkey kidney BSC40 monolayer cells by the calcium phosphate method (Zhang et al., 1992). Cells at 60-80% confluence were transfected in medium without phenol red and supplemented with 5% (v/v) charcoal-stripped fetal bovine serum. Transfections were carried out with 5 µg of luciferase reporter construct (pCPS*luc*, pHD(X3)*luc*, pM2(X3)*luc* or pAOx(X2)*luc*), 2 µg of each of RXRα and PPARα expression plasmids, and 0.5-4 µg of RZRα expression plasmid per 10 cm plate, as indicated. 2 µg of the β-galactosidase expression plasmid pCH110 (Pharmacia) was included in transfections as an internal reference. Effector plasmid dosage was kept constant by addition

of corresponding amounts of empty expression plasmids, and total DNA was maintained at 20 µg per 10 cm plate by addition of sonicated salmon sperm DNA. The peroxisome proliferator Wy-14,643 or the putative RZRα ligand melatonin (both in dimethyl sulfoxide) was added to fresh medium to a final concentration of 0.1 mM and 1 µM, respectively, where indicated. An equivalent volume of dimethyl sulfoxide was added to control medium. Cells were harvested and lysates prepared 48 h post-transfection, and luciferase activity was quantitated as previously described in Chapter 2 (Zhang et al., 1992).

5.4 RESULTS

5.4.1 RZRα binds as a monomer to the HD-PPRE but not the AOx-PPRE

Several potential RZRα consensus binding sites exist within the HD- and AOx-PPREs (Fig. 5-1), and interaction of RZRα with the HD-PPRE has recently been observed (Schrader et al., 1996). To explore how RZRα might cooperate with PPARα and RXRα to regulate transactivation from PPREs, we first examined the DNA-binding characteristics of these nuclear hormone receptors to the HD- and AOx-PPREs. EMSA was performed with radiolabelled HD- and AOx-PPREs and *in vitro* translated receptors. PPARα and RXRα formed a characteristic heterodimer that bound strongly to the HD-PPRE (Fig. 5-2*A*). RZRα also bound to the HD-PPRE, apparently as a monomer based on a comparison of the mobility of the RZRα complex with that of the heterodimeric PPARα/RXRα complex. Coincubation of RZRα with either PPARα or RXRα alone did not yield heterodimeric complexes. When all three receptors were present in the binding reaction, only heterodimeric PPARα/RXRα and

AOx-PPRE

CCGAACGTGACCTTTGTCCTGGTCCC

HD-PPRE

I II III IV

CCTCTCCTTTGACCTATTGAACTATTACCTACATT

RZRα Consensus Site

$TGACC(T/C)(A/T)_6$

Figure 5-1. Comparison of the sequence of the RZRα consensus binding site to sequences within the AOx- and HD-PPREs. Arrows and roman numerals indicate the locations and directions of TGACCT-like motifs.

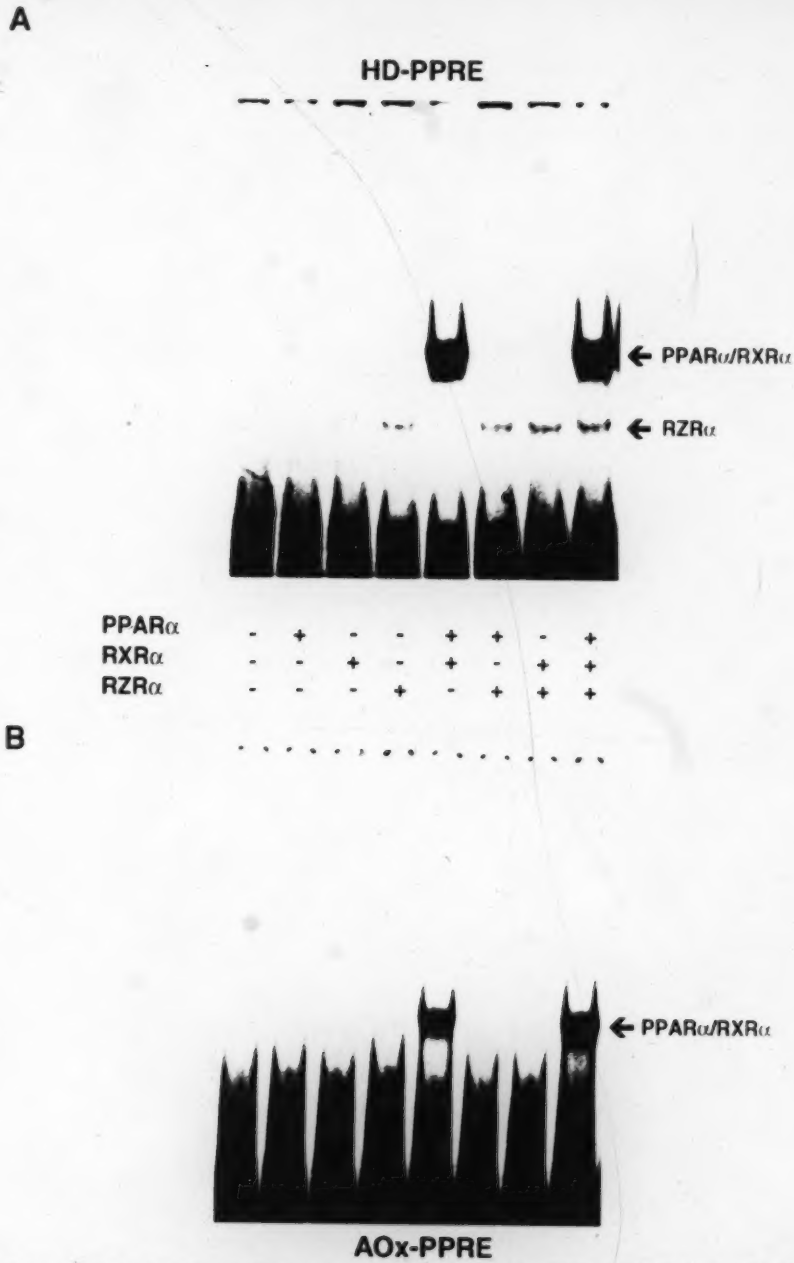


Figure 5-2. RZRα binds as a monomer to the HD-PPRE but not the AOx-PPRE. *In vitro* synthesized PPARα, RXRα and RZRα (1 μl each) were incubated alone or in combination with radiolabeled HD-PPRE (*A*) or AOx-PPRE (*B*) probe, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed as described under "Materials and Methods". The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by *arrows*.

monomeric RZRα complexes were observed, suggesting that these receptors do not co-occupy the HD-PPRE under these conditions. Therefore, RZRα binds as a monomer to the HD-PPRE and does not form complexes with PPARα or RXRα on the HD-PPRE *in vitro*. PPARα/RXRα heterodimers readily formed a complex on the AOx-PPRE, as expected; however, RZRα was unable to bind to this element either alone or in combination with PPARα or RXRα (Fig. 5-2*B*).

Competition analysis demonstrated that the interaction of RZRα with the HD-PPRE was specific. As shown in Fig. 5-3, both the RZRα and the PPARα/RXRα complexes were refractory to competition by nonspecific unlabeled oligonucleotide (compare *lanes d-f* to *lanes a-c, g*), whereas increasing amounts of unlabeled HD-PPRE oligonucleotide effectively competed for binding of both RZRα and PPARα/RXRα (compare *lanes h-j* to *lanes a-c, g*). These results show that binding of RZRα is specific for the HD-PPRE.

We next examined whether PPARα might modulate binding of RZRα to the HD-PPRE. EMSA was performed with radiolabelled HD-PPRE probe, constant amounts of RXRα and RZRα, and decreasing amounts of PPARα. Dilution of PPARα resulted in progressively decreased amounts of the PPARα/RXRα complex, as expected; however, there was no effect on formation of the RZRα monomer complex (Fig. 5-4*A*), suggesting that RZRα binding to the HD-PPRE *in vitro* is not influenced by PPARα. Similarly, increasing amounts of RZRα resulted in correspondingly greater amounts of the RZRα monomeric complex, but no change in the PPARα/RXRα complex (Fig. 5-4*B*). Additional analyses carried out using different amounts of radiolabelled HD-PPRE probe and titration of either RZRα (Fig. 5-5*A*) or PPARα/RXRα (Fig. 5-5*B*) showed that PPARα/RXRα and RZRα did

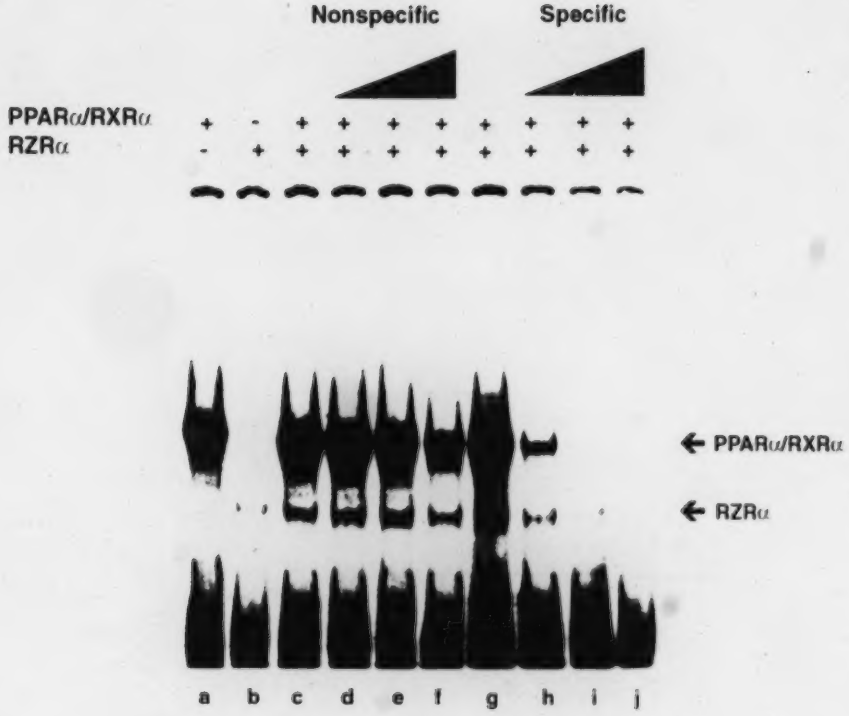
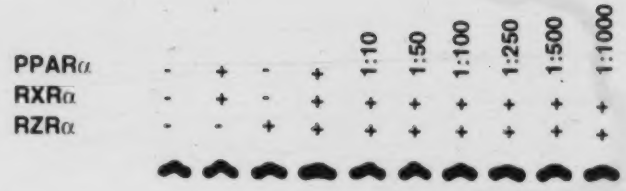
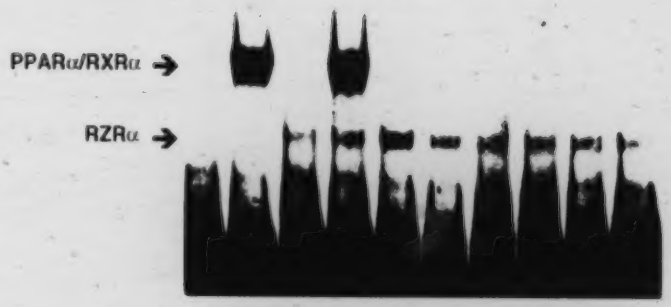


Figure 5-3. RZRα monomer binds to the HD-PPRE in a sequence-specific manner. EMSA was performed with *in vitro* synthesized receptors (1 μl each) and radiolabeled HD-PPRE probe, as shown. Volumes were kept constant by addition of unprogrammed lysate. Where indicated, unlabeled competitor DNA (5'-GATCCCGTGCATGCTAATGATATTCT and its complement) (*Nonspecific*) or unlabeled HD-PPRE (*Specific*) was present in 10-, 50- or 100-fold molar excess. The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by *arrows*.

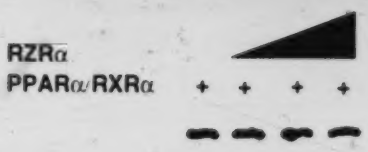
Figure 5-4. PPARα/RXRα and RZRα bind independently to the HD-PPRE. (*A*) *In vitro* synthesized RXRα and RZRα (1 μl each) were incubated with 1 μl of PPARα or increasing dilutions of PPARα in unprogrammed lysate, as shown, prior to addition of radiolabeled HD-PPRE probe. Complexes were analyzed by EMSA. The positions of PPARα/RXRα heterodimer and RZRα monomer complexes are indicated by *arrows*. (*B*) Radiolabeled HD-PPRE probe was incubated in the presence of constant amounts of *in vitro* synthesized PPARα and RXRα (1 μl each) and in the absence or presence of 1, 2 and 5 μl of *in vitro* synthesized RZRα. Lysate volumes were kept constant by addition of unprogrammed lysate. The positions of PPARα/RXRα heterodimer and RZRα monomer complexes are indicated by *arrows*.

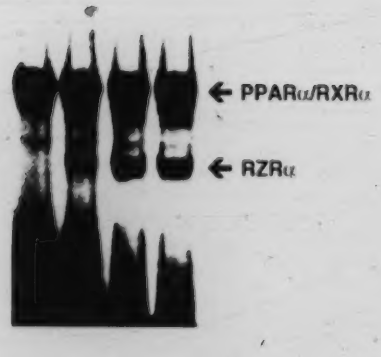
A


PPARα
RXRα
RZRα
1:10
1:50
1:100
1:250
1:500
1:1000


PPARα/RXRα
RZRα

B


RZRα
PPARα/RXRα


PPARα/RXRα
RZRα

not affect each other's binding to the HD-PPRE. Therefore, RZRα and PPARα/RXRα bind independently to the HD-PPRE.

5.4.2 RZRα and PPARα/RXRα bind to different regions of the HD-PPRE in vitro

The above results suggest that RZRα recognizes distinct determinants on the HD-PPRE compared to the requirements for PPARα/RXRα interaction. The HD-PPRE is a complex response element that consists of 4 consensus TGACCT hexameric half sites (sites I-IV; Fig.5-1). To determine which elements are responsible for RZRα binding specificity, oligonucleotide probes containing mutations in each of the four hexameric half sites were used in binding studies (Fig. 5-6). EMSA analysis showed that mutations in sites I, III, or IV did not adversely affect binding of RZRα, whereas disruption of site II eliminated binding (Fig. 5-6). This result is consistent with the fact that site II most closely matches the RZRα consensus sequence (TGACCT/C/(A/T)$_6$. By comparison, the integrity of sites III and IV were essential for PPARα/RXRα interaction, whereas sites I and II were dispensable (Fig. 5-6). This latter result is consistent with previous observations that the downstream DR1 element is necessary and sufficient for PPARα/RXRα signaling in the cell (Zhang et al., 1993). Thus, RZRα and PPARα/RXRα binding specificity is mediated by distinct and non-overlapping determinants in the HD-PPRE.

5.4.3 RZRα potentiates transactivation from the HD-PPRE by PPARα

To define a cellular role for the observed *in vitro* interaction of RZRα with the HD-PPRE, a series of transient transfections was performed with the HD-PPRE luciferase

Figure 5-5. RZRα and PPARα/RXRα do not affect each other's binding to the HD-PPRE. Binding reactions contained 2 µl of *in vitro* synthesized PPARα/RXRα and 0 µl, 2 µl or 5 µl of *in vitro* synthesized RZRα (*Panel A*); or 1 µl of *in vitro* synthesized RZRα and 0 µl, 4 µl or 10 µl of *in vitro* synthesized PPARα/RXRα (*Panel B*). Radiolabeled HD-PPRE probe was added to 200 fmol, 2 fmol or 0.2 fmol, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed as described in "Materials and Methods". Autoradiography was for 24 h (200 fmol) or 17 d (2 and 0.2 fmol).

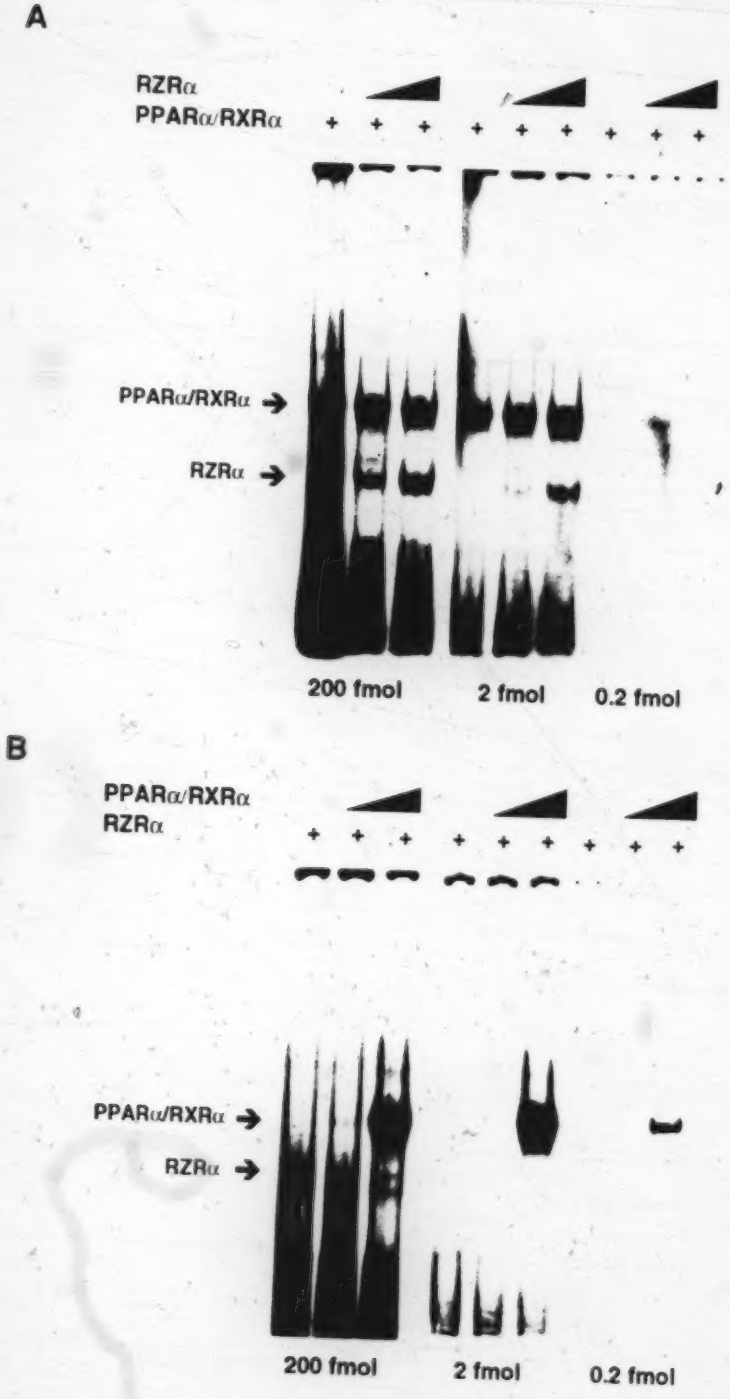
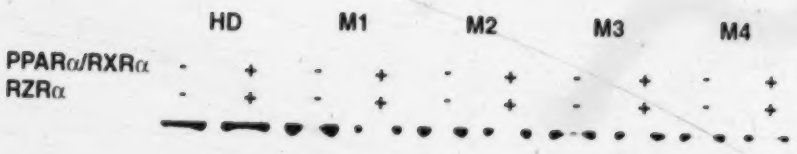

A
RZRα
PPARα/RXRα
PPARα/RXRα
RZRα
200 fmol
2 fmol
0.2 fmol
B
PPARα/RXRα
RZRα
PPARα/RXRα
RZRα
200 fmol
2 fmol
0.2 fmol

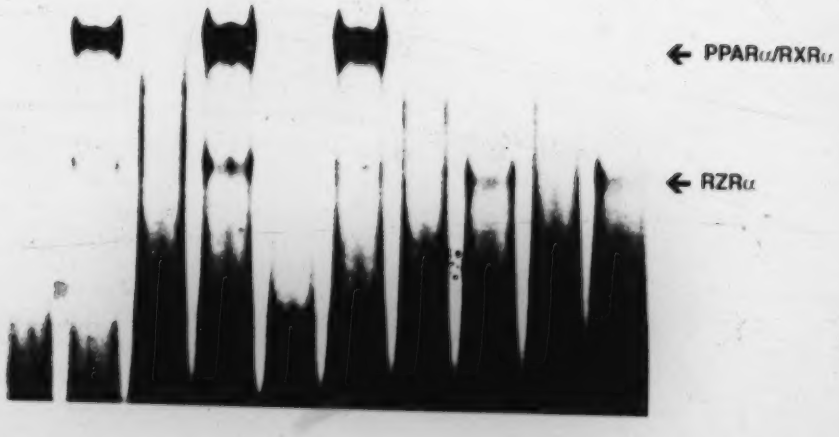
Figure 5-6. RZRα monomer and PPARα/RXRα heterodimer bind to different sites within the HD-PPRE *in vitro*. (*A*) Sequences of the wild-type HD-PPRE and of the oligonucleotides *M1*, *M2*, *M3* and *M4* containing mutations in consensus hexameric binding sites I, II, III and IV (*bold lettering*) of the HD-PPRE. *Open lettering*, sequences of mutant sites I, II, III and IV. (*B*) *In vitro* synthesized PPARα, RXRα and RZRα (1 μl each) were incubated with radiolabeled double-stranded oligonucleotides corresponding to mutations M1-M4. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed as described under "Materials and Methods". *Arrows* show the positions of bound complexes.

A

		I	II	III	IV
HD-PPRE	5' GATCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA				
M1	5' GATCCTATAATTTGACCTATTGAACTATTACCTACATTTGA				
M2	5' GATCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA				
M3	5' GATCCTCTCCTTTGACCTATTGAAGTATTACCTACATTTGA				
M4	5' GATCCTCTCCTTTGACCTATTGAACTAATCTTGACATTTGA				

B

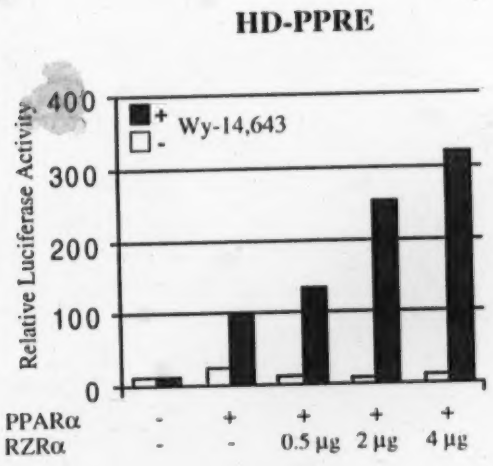




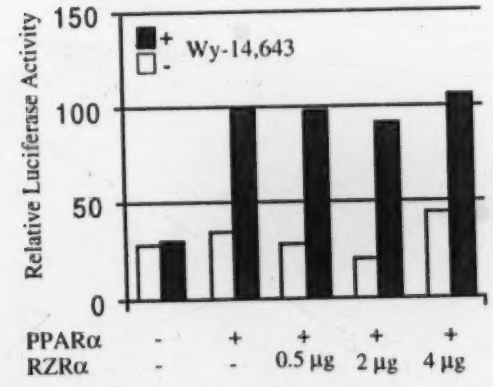
reporter plasmid, pHD(X3)*luc*, in BSC40 African monkey kidney cells. This cell line was chosen because it contains low levels of endogenous PPARα and RXRα activity (Miyata et al., 1993; Zhang et al., 1992). Transfection of a PPARα expression plasmid resulted in a 2-fold increase in luciferase activity over control transfections containing the reporter plasmid alone (Fig. 5-7*A*). Treatment of PPARα-expressing cells with the peroxisome proliferator and PPARα ligand, Wy-14,643, increased luciferase activity a further 4-fold . Transfection of increasing amounts of RZRα expression plasmid in the presence of fixed amounts of PPARα expression plasmid led to a dose-dependent increase in the level of luciferase activity in cells treated with Wy-14,643, but did not affect the ligand-independent response. At the maximal level of RZRα plasmid used (4 μg), ligand-dependent PPARα activity was 23 times the basal level of activity of control cells transfected with reporter plasmid alone (and compared to the 8-fold PPARα, ligand-dependent induction observed in the absence of RZRα expression plasmid). RZRα expression plasmid alone showed at most a 1.5-fold increase in luciferase activity over basal levels of the HD-PPRE luciferase reporter (Fig. 5-8), in agreement with previous studies that demonstrated that RZRα only weakly activates transcription via this element (Schrader et al., 1996). The modest increase observed reflects cooperation of RZRα with the HD-PPRE, as RZRα had no effect on basal expression levels of the parental pCPS*luc* reporter gene, which lacks a PPRE (see below). Importantly, functional cooperativity between PPARα and RZRα in transactivation from the HD-PPRE is abrogated in the reporter construct pM2(X3)*luc* (Fig. 5-7*B*), in which site II of the HD-PPRE required for binding of RZRα has been mutated. These results show that binding of RZRα to the HD-PPRE is apparently required to achieve transcriptional

Figure 5-7. RZRα potentiates transactivation by PPARα from the HD-PPRE but not the AOx-PPRE. BSC40 monolayer cells were transfected with 5 μg of the luciferase reporter pHD(X3)*luc* (*Panel A*), pM2(X3)*luc* (*Panel B*) or pAOx(X2)*luc* (*Panel C*), and expression plasmids for PPARα (2 μg) and RZRα (0.5-4 μg), in the absence or presence of 0.1 mM Wy-14,643. Plasmid dosage was normalized by addition of the appropriate empty vectors, where required. Cells were harvested 48 h post-transfection, and luciferase activity was quantitated, as described in Chapter 2. All values represent the average of three independent experiments carried out in duplicate. Values from individual transfections did not vary by more than 15%. The values are presented relative to the value obtained for cells transfected with PPARα in the presence of Wy-14,643 (taken as 100%).

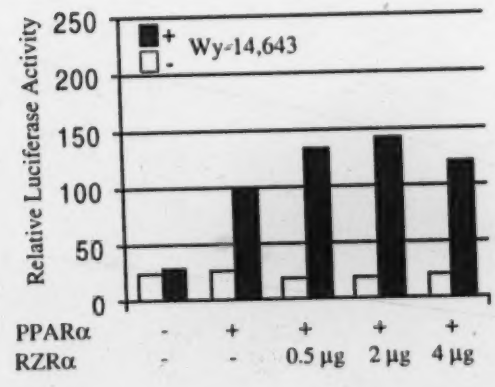
A


HD-PPRE
Relative Luciferase Activity
400
300
200
100
0
+ Wy-14,643
-
PPARα - + + + +
RZRα - - 0.5 µg 2 µg 4 µg

B


HD(M2)-PPRE
Relative Luciferase Activity
150
100
50
0
+ Wy-14,643
-
PPARα - + + + +
RZRα - - 0.5 µg 2 µg 4 µg

C


AOx-PPRE
Relative Luciferase Activity
250
200
150
100
50
0
+ Wy-14,643
-
PPARα - + + + +
RZRα - - 0.5 µg 2 µg 4 µg

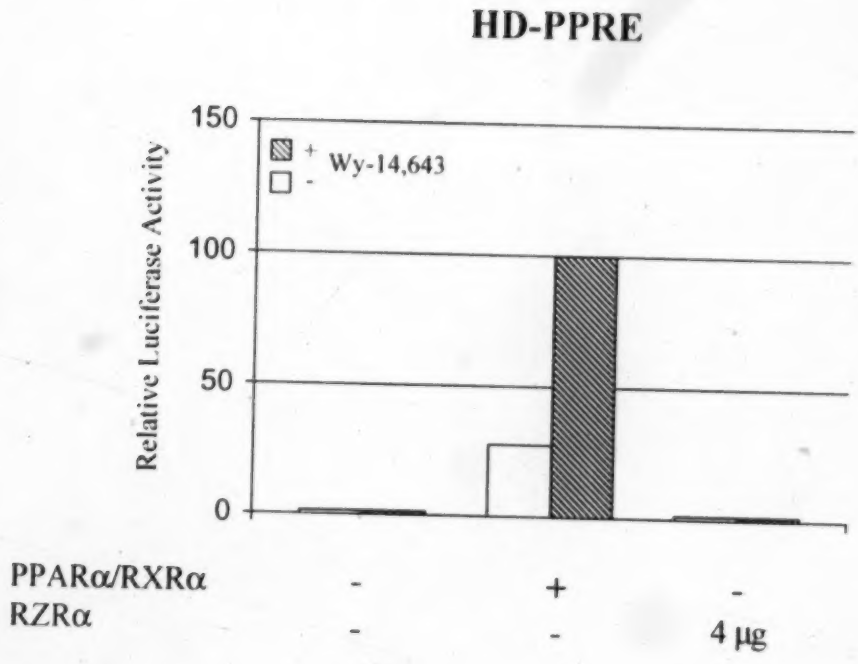


Figure 5-8. RZRα alone weakly activates transcription from the HD-PPRE. Transient co-transfections wre performed in BSC40 monolayer cells with 5 μg of pHD(X3)*luc* reporter plasmid, and expression plasmids for PPARα and RXRα (2 μg each) or RZRα (4 μg). Transfection media contained 0.1 mM of the peroxisome proliferator Wy-14,643 (in DMSO) or an equivalent volume of vehicle (DMSO). The data presented represent the average of three independent transfections performed in duplicate and values for individual transfections did not vary by more than 15%. The values are shown relative to the value obtained for cells transfected with PPARα/RXRα in the presence of Wy-14,643 (taken as 100%).

stimulation by PPARα.

The above experiments demonstrate that RZRα cooperates with PPARα to potentiate transactivation from the HD-PPRE in response to treatment with a peroxisome proliferator. In contrast, RZRα did not significantly affect PPARα-dependent transactivation from the AOx-PPRE, in either the absence or presence of ligand (Fig. 5-7*C*). These data are in keeping with the *in vitro* binding data presented above, and together the results demonstrate that RZRα binds specifically to the HD-PPRE and modulates transactivation by PPARα from this response element.

We next investigated whether melatonin, which has been suggested to be a putative ligand for RZRα (Carlberg and Wiesenberg, 1995; Steinhilber et al., 1995), could influence the functional cooperation between PPARα and RZRα in stimulating transcription from the HD-PPRE. Under our experimental conditions, we were unable to observe any effect of melatonin on the functional cooperativity of these two receptors in stimulating transcription from the HD-PPRE (Fig. 5-9).

5.4.4 Exogenous RXRα abrogates the potentiation effect of RZRα on transactivation by PPARα from the HD-PPRE

RXRα is an obligate heterodimerization partner for PPARα, and the PPARα-dependent activation presented above presumably arises from cooperation with low levels of endogenous RXRα present in BSC40 cells. We therefore examined the effects of RZRα under conditions where RXRα expression plasmid was included in the transfections. Coexpression of exogenous RXRα and PPARα increased luciferase activity 14- and 10-fold

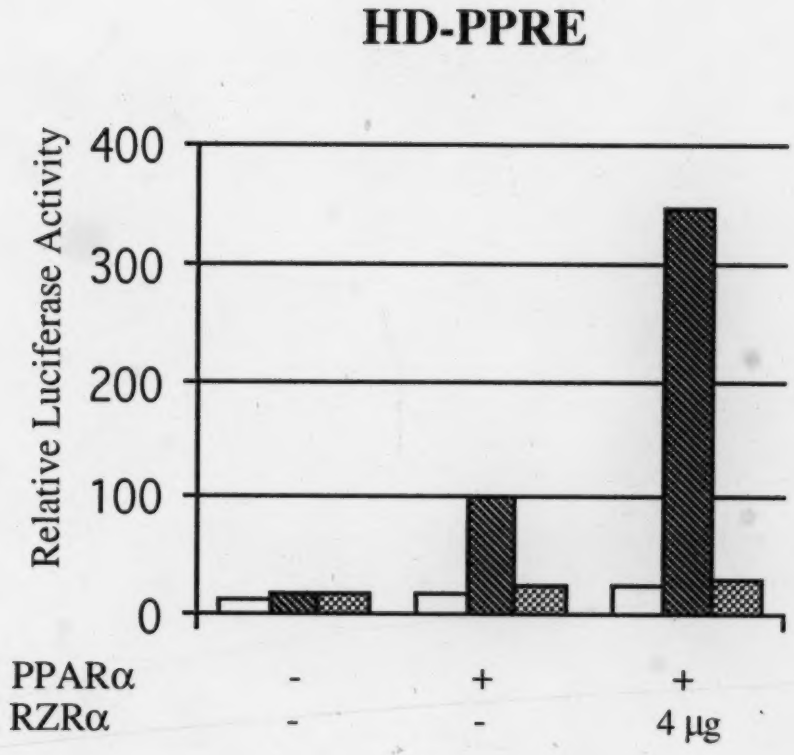
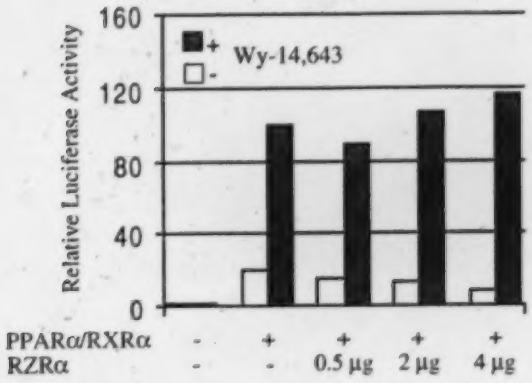


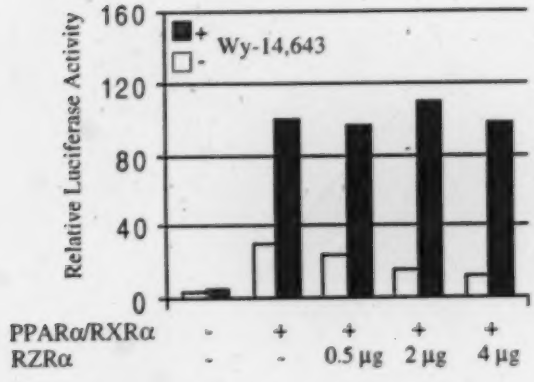
Figure 5-9. Melatonin does not stimulate transactivation by PPARα/RZRα from the HD-PPRE. BSC40 cells were transfected with 5 μg of the luciferase reporter pHD(X3)*luc* and the expression plasmids for PPARα (2 μg) and RZRα (4 μg), with addition of vehicle (dimethyl sulfoxide) (*open bars*), 0.1 mM Wy-14,643 (*hatched bars*) or 1 μM melatonin (*dashed bars*). Plasmid dosage was normalized by the addition of the appropriate empty vectors, where required. Cells were harvested 48 h post-trasnfection, and luciferase activity was quantitated. All values represent the average of three independent experiments carried out in duplicate. Values from individual transfections did not vary by more than 15%. The values are presented relative to the value obtained for cells transfected with PPARα in the presence of Wy-14,643 (taken as 100%).

Figure 5-10. RZRα does not potentiate transactivation by PPARα from the HD-PPRE in the presence of exogenous RXRα. BSC40 cells were transfected with plasmids expressing PPARα (2 μg), RXRα (2 μg) and RZRα (0.5-4 μg), and 5 μg of luciferase reporter construct. Values are shown for cells transfected with either (*A*) pHD(X3)*luc* or (*B*) pAOx(X2)*luc* reporter plasmids. The values are the average of three independent transfections performed in duplicate. Values from individual transfections did not vary more than 15%. The values presented were relative to the value obtained for cells transfected with PPARα and RXRα in the presence of Wy-14,643 (taken as 100%). (*C*) Transfection of BSC40 cells with the reporter plasmid pCPS*luc* in the presence or absence of PPARα (2 μg), RXRα (2 μg) and RZRα (0.5-4 μg), as indicated. Values represent the average of duplicate transfections.

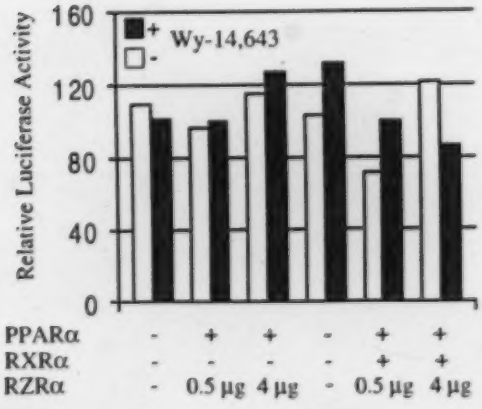
A **HD-PPRE**



B **AOx-PPRE**



C **pCPS*luc***



over basal levels for the HD-PPRE (Fig. 5-10*A*) and AOx-PPRE (Fig. 5-10*B*) luciferase reporter plasmids, respectively. In the presence of Wy-14,643, activity was increased 60-fold for the HD-PPRE reporter (Fig. 5-10*A*) and 20-fold (Fig. 5-10*B*) for the AOx-PPRE reporter. The robust activity obtained with coexpressed RXRα is consistent with the fact that the endogenous level of this receptor is limiting for PPARα-mediated transactivation. Interestingly, under these conditions, transactivation from the HD- and AOx-PPRE reporter plasmids was not significantly affected by addition of RZRα (Figs. 5-10*A* and *B*, respectively). Control transfections with the parental construct pCPS*luc*, which lacks a PPRE, showed that the presence of PPARα, RXRα, and RZRα did not influence the levels of relative luciferase activity observed (Fig. 5-10*C*), indicating that these receptors did not alter the basal activity of the parental reporter construct. Together, these data suggest that the stimulatory effect observed with RZRα on transactivation by PPARα is attenuated by increasing the level of RXRα.

5.5 DISCUSSION

Several observations have suggested a degree of interplay between the RZR/ROR and PPAR nuclear hormone receptor families in the regulation of genes, particularly those encoding proteins involved in lipid metabolism. For instance, both PPARα and RZRα regulate transcription of the apolipoprotein AI gene (Vu-Dac et al., 1997; Vu-Dac et al., 1994). PPARγ has been shown to be a critical regulator of the adipogenic program, and RORα and RORγ mRNAs are induced early in adipogenesis (Austin et al., 1998). Finally, the antidiabetic thiazolidinediones, which are potent activators of PPARγ, have recently been

shown to also be specific ligands for RZRα (Lehmann et al., 1995; Missbach et al., 1996).

Both the HD and AOx genes are regulated by a number of nuclear hormone receptors from several different signaling networks that converge on the respective PPREs or that directly modulate PPARα activity (Miyata et al., 1996; Miyata et al., 1993; Winrow et al., 1996; Winrow et al., 1994). Consistent with these observations, Schräder *et al.* reported that RZRα weakly interacted with the HD-PPRE and was able to minimally activate transcription via this element (Schrader et al., 1996). Our results extend this finding to show that RZRα can strongly potentiate transactivation by PPARα from the HD-PPRE when RXRα levels are limiting. We found that the monomeric binding of RZRα to the HD-PPRE was specific and required for potentiation of transactivation by PPARα from the HD-PPRE, and that RZRα did not bind to, or stimulate PPARα-dependent transactivation from, the AOx-PPRE.

The mechanism by which RZRα stimulates PPARα activity on the HD-PPRE is not known. EMSA analysis did not show any obvious antagonizing or stabilizing effects between RZRα monomers and PPARα/RXRα heterodimers on the HD-PPRE *in vitro*. This result agrees with our findings that the RZRα monomer and PPARα/RXRα heterodimer target distinct and non-overlapping hexameric determinants for binding to the HD-PPRE. Moreover, we did not observe a higher order ternary complex containing PPARα/RXRα/RZRα on the HD-PPRE. However, this does not preclude the possibility of such a complex forming *in vivo*, which may be dependent on cooperativity or interaction with auxiliary cofactors. The involvement of auxiliary factors such as SRC-1, p300 and N-COR in transcriptional regulation by PPARs and other nuclear hormone receptors is well established (DiRenzo et al., 1997; Dowell et al., 1997b; Zhu et al., 1996). Additionally,

PPARα, RXRα and/or RZRα might require phosphorylation or other modification not provided in the *in vitro* transcription/translation system. We are currently investigating whether auxiliary factors and receptor post-translational modification affect cooperative transcriptional regulation by PPARα, RXRα and RZRα.

The HD-PPRE is a complex response element and among a select few that contain 4 hexameric direct repeats. In the HD-PPRE, these hexameric half sites are organized in two tandem DR1 arrays that are separated by 2 nucleotides, an arrangement that is thought to facilitate diverse receptor interactions and thereby permit multiple levels of control. A model has been proposed in which either one or two PPARα/RXRα heterodimers bind to the HD-PPRE to determine the state of transcriptional activation (Chu et al., 1995a). As we have shown previously, the 3' DR1 (sites III/IV) array is essential and sufficient for PPARα/RXRα binding and activity (Zhang et al., 1993); however, the arrangement that has been suggested to yield the highest level of transactivation has PPARα/RXRα heterodimers bound to both DR1 sites. Based on our observations *in vitro*, it is likely that under certain *in vivo* conditions, such as limited availability of RXRα, a single PPARα/RXRα heterodimer may bind to the second DR1 element (sites III/IV), yielding a lower overall level of transactivation compared with the situation in which two PPARα/RXRα bind to this PPRE. Since RZRα occupies site II within the HD-PPRE, this may preclude binding of PPARα/RXRα to the first DR1 element and thereby favor binding of the heterodimer to the transcriptionally competent 3' DR1 array. RZRα may also contribute directly to transcriptional responses by stabilizing the PPARα/RXRα complex *in vivo* or facilitating interactions with an auxiliary factor(s). This pathway of stimulation may be operative when RXRα is present in limiting amounts, for

example in transfections carried out with PPARα alone, as RZRα had no effect when transfections were carried out in the presence of excess exogenous RXRα. Cotransfection of RXRα significantly increased the overall level of transactivation by PPARα, as one would expect if endogenous RXRα is limiting. It is possible that this level of activity is beyond a threshold level at which RZRα might be expected to have a stimulatory effect. Moreover, when RXRα is present in excess *in vivo*, PPARα/RXRα heterodimers may occupy both DR1s, thereby resulting in a maximal transcriptional response and preventing RZRα from accessing site II. This model suggests ligand-mediated regulation of the HD-PPRE is dependent on input and dynamic interplay among the PPARα, RXRα and RZRα signaling pathways.

In summary, these findings demonstrate that the orphan receptor RZRα can work in cooperation with PPARα to regulate expression of the gene encoding HD, the second enzyme of the peroxisomal β-oxidation pathway. Transcriptional regulation of peroxisomal β-oxidation has proven to be a dynamic process, integrating cues from a host of signaling pathways and rapidly responding to variations in levels of key components. An understanding of the principal regulators of peroxisomal β-oxidation, including transcription factors controlling and modulating the expression of the genes encoding the enzymes of this pathway, may provide for the development of pharmacologic agents that specifically target the peroxisomal β-oxidation system as a means to influence overall lipid metabolism and homeostasis.

CHAPTER 6

GENERAL DISCUSSION

6.1 Discussion

Currently the most prevalent diseases in our society are directly related to the control of lipid metabolism and homeostasis. Fatty acids serve not only as energy stores, but also as critical signalling molecules, altering gene expression, and eliciting widespread effects on metabolism, cell growth and differentiation (for reviews see Clarke and Jump, 1994; Jump et al., 1997). The influences of dietary fat have been examined in the initiation and progression of numerous chronic diseases including cancer, insulin resistance, hyperlipidemia, cardiovascular disease, obesity and hypertension (Hallaq et al., 1992; Pan et al., 1994; Phillipson et al., 1985; Storlien et al., 1991; Welsch, 1992). A better understanding of the core regulatory mechanisms which control fatty acid metabolism will provide additional targets for genetic screening and will direct the discovery of pharmacological agents which can be employed to ameliorate a variety of disease states. Nuclear receptors are critical regulators of fatty acid metabolism and provide excellent targets for pharmacological intervention since the majority are ligand-modulated and since they serve as specific activators and/or repressors of target genes. Members of the PPAR family of nuclear receptors were initially identified as regulators of peroxisomal β-oxidation and time has shown that these factors play an exceedingly important part in mounting the necessary responses to fluctuating fatty acid levels and maintaining the balance between lipid storage and metabolism. My research has focussed on the identification of factors which modify the activity of PPARs in response to peroxisome proliferators, with a particular eye towards the transcriptional regulation of enzymes of the peroxisomal fatty acid β-oxidation pathway. The results I have presented clearly demonstrate the role of several factors in the PPAR-mediated transactivation

of the rat AOx and HD genes which can be extended to other species, and add to the general understanding of nuclear receptor signalling and the part played by additional signalling molecules.

6.1.1 Influence of HNF-4 on the AOx- and HD-PPREs

HNF-4 regulates genes involved in lipid metabolism, exhibits a similar tissue distribution as PPARα and recognizes response elements with DR1 spacing (Dreyer et al., 1992; Fraser et al., 1998; Gregori et al., 1998; Hall et al., 1995; Jiang et al., 1995; Murao et al., 1997; Sladek et al., 1990; Stoffel and Duncan, 1997). In our work with HNF-4, we observed that HNF-4 interacted readily with the AOx-PPRE and to a lesser extent with the HD-PPRE. We found that HNF-4 repressed PPARα activation of the AOx- and HD-PPRE in the absence of a peroxisome proliferator. Interestingly, when a peroxisome proliferator was included in transient co-transfections, HNF-4 repressed the AOx-PPRE and potentiated the activation of the HD-PPRE by PPARα. A recent study has confirmed our findings regarding the inhibition of PPARα-mediated transactivation of the AOx gene by HNF-4 through direct competition for binding to the AOx-PPRE (Nishiyama et al., 1998). Our demonstration that HNF-4 differentially regulates the PPARα-dependent transactivation of the AOx- and HD-PPRE was the first example that HNF-4 can function as both a transcriptional activator and repressor of lipid metabolism through a common response element. This research identifies HNF-4 as an important transcriptional regulator of peroxisomal β-oxidation and shows the interplay between the HNF and PPAR signalling networks.

The observation that HNF-4 represses Wy-14,643- independent transactivation of the AOx- and HD-PPREs and Wy-14,643-dependent transactivation of the AOx-PPRE can be accounted for by competition for DNA binding site occupancy by HNF-4 homodimers. This repression may also involve the formation of non-DNA binding HNF-4 heterodimers and may occur through interactions between the ligand binding domain-dimerization interfaces in the absence of DNA binding domain-dimerization interface interactions. Although this is possible, it may not be the case, since HNF-4 has only been reported to function as a homodimer (Jiang et al., 1995).

Since our initial examination of the interactions between HNF-4 and PPARα/RXRα signalling, a number of studies have been performed which have investigated the cross-talk between these nuclear receptor families. HNF-4 and PPAR interact as both activators and as repressors of co-regulated genes (Hertz et al., 1995; Hertz et al., 1996b; Murao et al., 1997; Nishiyama et al., 1998; Peters et al., 1997b; Rodriguez et al., 1994; Rodriguez et al., 1998). Response elements in the promoters of the long terminal repeat of HIV-I, the hepatitis B virus nucleocapsid gene, phosphoenolpyruvate carboxykinase, fatty acid binding protein and cellular retinol binding protein genes, are all synergistically activated through the co-operation of HNF-4 and PPARs (Hall et al., 1995; Kliewer et al., 1992b; Ladias, 1994; Poirier et al., 1997; Raney et al., 1997; Stoffel and Duncan, 1997; Tontonoz et al., 1995). In addition to AOx and HD, HNF-4 and PPARα have been subsequently found to differentially regulate expression of the liver transferrin and apolipoprotein CIII genes and the hepatitis B virus pre-C promoter (Fraser et al., 1998; Hertz et al., 1995; Hertz et al., 1996b; Yu and Mertz, 1997).

Early studies of the regulation of the apolipoprotein CIII gene showed that although

HNF-4 served as a transcriptional activator of this gene, fibrate peroxisome proliferators suppressed apolipoprotein CIII gene transcription (Fraser et al., 1998; Hertz et al., 1996a; Hertz et al., 1995). It was determined that PPARα competes with HNF-4 for binding to apolipoprotein CIII response elements and displaces the transactivating HNF-4 (Hertz et al., 1996a; Hertz et al., 1995). In addition, peroxisome proliferators repress expression of the HNF-4 gene (Hertz et al., 1996b). The interplay between HNF-4 and PPARα/RXRα in the regulation of the apolipoprotein CIII gene is thought to partially account for the hypolipidemic effects of peroxisome proliferators (Hertz et al., 1995; Hertz et al., 1996b). Apolipoprotein CIII inhibits the catabolism of triglyceride-rich lipoproteins by the inhibition of lipoprotein lipase and by blocking liver receptor mediated uptake (Hertz et al., 1996a; Hertz et al., 1995). Therefore, peroxisome proliferator inhibition of apolipoprotein CIII expression coupled with increases in β-oxidation might account for an overall decrease in plasma lipid levels (Hertz et al., 1996a; Hertz et al., 1995).

HNF-4 represses PPARα transactivation of the mitochondrial HMG-CoA synthase gene, illustrating another example of differential control by these nuclear receptors (Rodriguez et al., 1994; Rodriguez et al., 1998). These observations reiterate the importance of the interplay between the HNF-4 and PPAR signalling pathways which we first described in the differential regulation of the AOx- and HD-PPREs. Prior to our investigation, HNF-4 had been shown to stimulate expression of mitochondrial medium chain acyl-CoA dehydrogenase, which is a key enzyme of the mitochondrial β-oxidation pathway (Carter et al., 1993). Our results indicate that HNF-4 represses the transcription of the peroxisomal β-oxidation AOx and HD genes in the absence of a peroxisome proliferator. Taken together,

these findings suggest that HNF-4 may serve as a molecular switch to direct activation of mitochondrial β-oxidation, while down regulating peroxisomal β-oxidation. It appears, however, that these interactions may be more complex than first thought, since PPARα has also been shown to activate the mitochondrial medium chain acyl-CoA dehydrogenase gene in response to peroxisome proliferators (Gulick et al., 1994).

Recent evidence supports the role of cross-talk between PPAR and HNF-4 receptors on a number of fronts. Hypolipidemic peroxisome proliferators reduce the expression of the HNF-4 gene, and this may prove effective in modulating cross-talk between the PPAR and HNF-4 pathways (Hertz et al., 1996b). Interplay between these receptors is not unique to the AOx and HD genes, since HNF-4 and PPARα differentially regulate expression of the apolipoprotein CIII, HMG-CoA synthase and liver transferrin genes and the hepatitis B virus pre-C promoter (Hertz et al., 1996a; Hertz et al., 1996b; Rodriguez et al., 1998; Yu and Mertz, 1997).

Perhaps the most striking indication of the convergence of the PPAR and HNF-4 signalling networks is that fatty acyl-CoA thioesters with chain lengths greater than C12 act as ligands for HNF-4 (Hertz et al., 1998). Saturated C14 or C16 fatty acyl-CoAs serve as agonists, potentiating HNF-4 homodimerization, DNA binding and gene activation. C18 and polyunsaturated fatty acyl-CoAs act as antagonists by increasing the formation of non-DNA binding HNF-4 homodimers, resulting in a significant decrease in transcription of target genes. The fatty acid chain length and degree of saturation appear to determine whether the transcriptional effects will be activating or repressive (Hertz et al., 1998). PPARs are activated by mono- and polyunsaturated long chain free fatty acids, including those with chain

lengths greater than C16 (Bentley et al., 1993; Dreyer et al., 1993; Forman et al., 1997a; Kliewer et al., 1997; Krey et al., 1997; Willson and Wahli, 1997). These findings imply a mechanism for synergistic and antagonistic cross-talk between PPARs and HNF-4 nuclear receptors at the level of ligand binding and availability (Hertz et al., 1998). In addition, peroxisomal β-oxidation preferentially metabolizes very long chain fatty acids, with chain lengths between C18-C30 (Hiltunen et al., 1996; Mannaerts and van Veldhoven, 1996; Mannaerts and Van Veldhoven, 1993; Reddy and Lalwai, 1983).

Coupled with our results, these findings implicate HNF-4 as an extremely important mediator of fatty acid metabolism, interacting with the peroxisomal proliferator signalling pathway to direct either mitochondrial or peroxisomal β-oxidation of fatty acids. This holds implications for HNF-4 and PPARα interplay in the monitoring of physiological lipid levels and stimulation of fatty acid metabolism via β-oxidation. It will be interesting to determine more specifically how the PPAR and HNF-4 signalling pathways converge, and to characterize these interactions within the context of a whole animal. Given the discovery of an increasing number of genes that are co-regulated by HNF-4 and PPARα, the interplay between these nuclear receptors will likely become more apparent in other critical processes such as mitochondrial ω-hydroxylation, the inflammatory response, and the activation of viral genes (Aldridge et al., 1995; Huss and Kasper, 1998; Ladias, 1994; Muerhoff et al., 1992; Peters et al., 1997b; Raney et al., 1997; Ueda et al., 1998; Yokomori et al., 1997; Yu and Mertz, 1997).

6.1.2 Calreticulin and PPARα/RXRα activity

Calreticulin is a Ca^{2+}-binding protein which exerts effects on cell adhesion, gene expression, and signal transduction, and functions as an endoplasmic reticulum chaperone responsible for protein folding and processing (Coppolino and Dedhar, 1998; Krause and Michalak, 1997; Mery et al., 1996; Opas et al., 1996; Vassilakos et al., 1998). Based on initial observations with glucocorticoid, retinoic acid, vitamin D3 and androgen receptors, a number of groups had proposed that calreticulin might generally regulate nuclear receptor activity through interactions with the conserved KXXFF(K/R)R motifs in their DNA binding domains (Burns et al., 1994; Dedhar et al., 1994; St-Arnaud et al., 1995; Wheeler et al., 1995). The research that we have performed demonstrated that although calreticulin could reduce the *in vitro* DNA binding of COUP-TF, HNF-4 and PPARα/RXRα, the effects of calreticulin overexpression on PPARα/RXRα-mediated gene activation were negligible in a cellular context. We also observed repression of GR-mediated gene expression as has been previously reported.(Burns et al., 1994) Our experiments were the first demonstration that calreticulin is not a global regulator of nuclear receptor transactivation, and these findings have been strengthened in light of more recent findings.

In order to directly affect nuclear receptor function through physical interactions with the KXXFF(K/R)R sites within the nuclear receptor DNA-binding domains, calreticulin would need to be present outside of the endoplasmic reticulum. It had been originally reported that calreticulin is found in several locations including the cell surface, within the secretory pathway, in the cytoplasm and within the nucleus (Dedhar et al., 1994; Dupuis et al., 1993; Michalak et al., 1992; White et al., 1995). Since that time, the localization of

calreticulin outside of the endoplasmic reticulum has been questioned (Krause and Michalak, 1997; Michalak et al., 1996). Using a combination of separately derived antibodies towards native calreticulin as well as recombinant dystrophin- and green fluorescent protein-epitope tagged calreticulin, Opas concluded that calreticulin is localized exclusively to the endoplasmic reticulum network and is not a constitutive nuclear protein (reviewed in Krause and Michalak, 1997). Extensive immunohistological work in several cell types and with a number of calreticulin antibodies, and with both intact and fractionated nuclei has been conducted (Michalak et al., 1996). The conclusion of these studies was that calreticulin is not present as a nuclear protein. Use of a cytoplasmically targeted calreticulin mutant showed that only overexpression of the native endoplasmic reticulum-resident calreticulin was able to affect GR-mediated gene expression (Michalak et al., 1996). In addition, when GR is bound to DNA, calreticulin is unable to compete for GR binding, suggesting that the DNA-bound nuclear form of GR does not interact with calreticulin (Michalak et al., 1996). These findings imply that calreticulin is not interacting directly with the KXXFF(K/R)R motifs within the DNA-binding sites of nuclear receptors in the nucleus and thereby disrupting gene activation (Krause and Michalak, 1997; Michalak et al., 1996). Due to the nature of these experiments, however, one cannot completely exclude the possibility that calreticulin is present transiently in the nucleus. The weight of this evidence suggests that the effects of calreticulin on gene expression are occurring through other means, possibly by endoplasmic reticulum signalling or through alteration of Ca^{2+}-signalling (Krause and Michalak, 1997; Opas et al., 1996).

It is known that calreticulin can interact with factors within the endoplasmic reticulum and may be modulating nuclear receptor activity through associations with endoplasmic

reticulum-resident signalling molecules. Protein disulfide isomerase is localized to the endoplasmic reticulum and differentially interacts with calreticulin in response to Ca^{2+}-binding (Baksh et al., 1995). Calreticulin is a critical regulator of Ca^{2+} stores within the endoplasmic reticulum lumen and also appears to govern Ca^{2+} levels in the nuclear envelope and cytoplasm (Krause and Michalak, 1997; Mery et al., 1996; Zhu et al., 1998). As a key regulator of Ca^{2+} levels, calreticulin may affect nuclear receptor activity by altering transport of molecules across the nuclear membrane and could have upstream effects on Ca^{2+}-mediated nuclear receptor signalling and/or receptor localization. Regulatory interactions between calreticulin and nuclear receptors are very plausible when considering the role of calreticulin in regulating Ca^{2+} stores (Mery et al., 1996).

There is precedent for the involvement of nuclear receptors in the regulation of, and response to changes in intracellular Ca^{2+} levels, and in the mediation of cross-talk between hormonal and Ca^{2+}-signalling networks. Among the nuclear receptors with demonstrated roles in Ca^{2+} homeostasis and/or Ca^{2+}-signalling are the vitamin D3, estrogen and glucocorticoid receptors (Carlberg and Polly, 1998; Dennis et al., 1987; Yamaguchi and Oishi, 1995). Our laboratory and others have demonstrated that the transcriptional activities of VDR and GR are dramatically repressed by overexpression of calreticulin (Burns et al., 1994; Dedhar et al., 1994; Krause and Michalak, 1997; St-Arnaud et al., 1995; Wheeler et al., 1995; Winrow et al., 1995). Interestingly, the peroxisome proliferators DHEA-S and 5-androstene-3-β-17 β-diol, and the PPARα ligand leukotriene B_4 are linked to changes in intracellular Ca^{2+} levels (Waxman, 1996; Yokomizo et al., 1997). The interplay between calreticulin and nuclear receptor-directed gene expression appears to involve aspects of Ca^{2+}-

signalling and not direct physical interactions between calreticulin and conserved motifs in the DNA binding domains of nuclear receptors. An understanding of what role calreticulin is playing in gene expression is currently an area of intense investigation, however the precise molecular mechanisms involved remain to be determined.

The findings that we have reported provide opportunities for future studies to determine the mechanisms by which calreticulin is affecting the activity of certain nuclear receptors. It will be interesting to ascertain which other nuclear receptors are unaffected or possibly activated by increased calreticulin levels and to compare these receptors with PPARα and RXRα in order to better understand how calreticulin is affecting gene expression. An additional interesting area to investigate is whether calreticulin is capable of modulating the activity of PPARδ or PPARγ. The differences in nuclear receptor susceptibility to calreticulin control could be due to the Ca^{2+}-signalling requirements of each particular nuclear receptor. The mechanisms by which endoplasmic reticulum signalling occur are not well understood, but as they become more apparent they will yield greater insight into new means by which signal transduction by the nuclear receptor and other signalling pathways are regulated. This will undoubtedly result in the identification of novel signalling factors and illustrate the co-integration of distinct signalling pathways responsible for directing gene expression.

6.1.3 The Effects of TRα on Peroxisome Proliferator Signalling

The thyroid hormone receptor (TR) family is a well studied group of nuclear receptors whose members are responsible for directing a wide variety of cellular processes, including lipid metabolism and homeostasis (Goodridge, 1987; Shepard and Eberhardt, 1993; Silva and

Rabelo, 1997; Xiong et al., 1998). Most recently, examinations of TR functions have illustrated general concepts for other nuclear receptors in terms of interactions with co-activators and co-repressors (Chen and Evans, 1995; Fondell et al., 1996; Lee et al., 1995; Wagner et al., 1995; Yuan et al., 1998). We observed that TRα binds to the AOx-PPRE as both a monomer and as a TRα/RXRα heterodimer. We further demonstrated that unliganded TRα augmented transactivation by PPARα from the AOx-PPRE in the presence of a peroxisome proliferator. When activated by its ligand, T3, TRα potently repressed transactivation of the AOx-PPRE by PPARα.

Following our illustration that TRα modulates peroxisome proliferator-directed expression of the AOx-PPRE, two other groups showed interplay between TR, PPAR and RXR in mediating expression from this element (Chu et al., 1995b; Miyamoto et al., 1997) There have also been a number of additional findings which illustrate cross-talk between thyroid hormone and peroxisome proliferator signalling networks in modulating expression of target genes. These include the co-regulation of the malic enzyme gene, microsomal NADPH:cytochrome P450 oxidoreductase, CYP 4A1, 4A2 and 4A3, and uncoupling protein genes (IJpenberg et al., 1997; Silva and Rabelo, 1997; Webb et al., 1996). The interplay between the TR and PPAR families that is evident in the regulation of genes involved in lipid metabolism and differentiation, may also overlap in governing other key processes such as apoptosis (Chinetti et al., 1998; Christensen et al., 1998; Llanos et al., 1998; Nagasawa et al., 1997; Tata, 1994). An understanding of the roles of co-activators/co-repressors, dimerization requirements, chromatin remodelling, DNA core site recognition and receptor agonists and antagonists in co-regulation by TR, RXR and PPAR will evolve through examinations of

common target response elements, similar to those we have conducted with the AOx-PPRE.

Subsequent to our demonstration of PPARα, RXRα and TRα interplay, it was reported that TRα inhibits peroxisome proliferator-induced transactivation of the AOx-PPRE in CV-1, H4IIEC3 and COS-1 cells both in the presence and absence of T3 (Chu et al., 1995b; Miyamoto et al., 1997). The differences between our findings and those presented by these groups may be due to several cell specific factors. We employed the BSC40 African green monkey kidney cell line derived from BS-C-1 cells for transient transfections (Hopps et al., 1963). It is possible that differences in the endogenous levels of nuclear receptors and/or accessory factors may contribute to the differences observed. It would be interesting to examine to what degree known PPARα/RXRα interacting co-repressors, such as SMRT, are present in BSC40 cells and to compare the expression levels of these factors in CV-1, H4IIEC3 and COS-1 cells. Some nuclear receptor accessory factors demonstrate a restricted tissue distribution and this may account for one of the means by which tissue specific gene expression is realized (Jain et al., 1998; Misiti et al., 1998; Smith et al., 1997). Additionally, the differences in the observed effects of unliganded TRα on the AOx-PPRE may be due to differences in the expression or endogenous levels of PPARα, RXRα or TRα in the cell lines employed. One of the reasons for using the BSC40 cells is that this cell line contains low levels of intrinsic PPAR, RXR and TR activity, however some residual RXR activity is observed. Since competition for RXRα is proposed to be a major means by which TR affects PPAR-signalling (Chu et al., 1995b; Juge-Aubry et al., 1995), the relative abundance of RXRα in the cell lines examined must be taken into consideration.

TRα can function as a monomer, homodimer and as a heterodimer (Desvergne, 1994;

Ikeda et al., 1994; Lazar et al., 1991b; Tsai and O'Malley, 1994). Although we observe binding of TRα monomers and TRα/RXRα heterodimers to the AOx-PPRE *in vitro*, it may be that non-DNA binding TRα dimers are also contributing to the overall transactivation from this response element *in vivo*. TRα heterodimerizes with RXRα and it is reported that TRα/RXRα heterodimers have less restrictive DNA-binding requirements than TRα homodimers (Olson and Koenig, 1997). TRα was observed to decrease the binding of PPARα/RXRα to the AOx-PPRE and this is likely due in part to the sequestration of RXRα and competition of TRα/RXRα heterodimers for the binding to the AOx-PPRE. These mechanisms have been suggested to explain TRα actions with the AOx-PPRE since our initial investigation (Chu et al., 1995b; Miyamoto et al., 1997). TRα can also sequester PPARα in solution, and this may also account for the reduced PPARα/RXRα binding to the AOx-PPRE which we observed (Bogazzi et al., 1994). It appears that, contrary to what is reported for TRα/RXRα heterodimers, TRα/PPARα heterodimers have a much more restricted DNA-binding preference, possibly as a result of the associations between their heterodimerization interfaces.

One explanation for the TR-mediated potentiation of the AOx-PPRE by PPARα/RXRα could be the sequestration of a shared co-repressor, such as SMRT, by TRα. In the absence of ligand, TRs act as potent transcriptional silencers (Baniahmad et al., 1992; Burcin et al., 1994; Nawaz et al., 1995; Renkawitz, 1993). Analysis of this observation showed that the transcriptional silencing by TRs is dependent on interactions with co-repressors (Baniahmad et al., 1995; Chen and Evans, 1995; Kurokawa et al., 1995; Lee et al., 1995; Sande and Privalsky, 1996). It has been clearly demonstrated that unliganded TRα

associates with co-repressors and it is possible that TRα which is not bound to DNA may be sequestering co-regulatory factors which would otherwise interact with PPARα or RXRα (Chen and Evans, 1995; Kurokawa et al., 1995; Lee et al., 1995). Such interactions would facilitate the association of liganded PPARα/RXRα heterodimers with positive co-activators, with the net result being an increase in the transactivation by PPARα/RXRα.

In the presence of the T3, transactivation of the AOx-PPRE is potently inhibited when TRα is included in transfections. There may be several reasons for this, including the titration of common co-activators from PPAR/RXR, formation of ligand-directed non-DNA binding TRα/PPARα or TRα/RXRα heterodimers, or binding of liganded TRα monomers or TRα/RXRα heterodimers to the AOx-PPRE in a conformation which represses transcription. TRα is functional in our system, since it is able to activate transcription of a thyroid response element in response to T3. Although it is more commonly observed that ligand binding increases transactivation, liganded nuclear receptors can also function as transcriptional repressors depending on the promoter context. This phenomenon has been observed in PPARα/RXRα interactions with response elements of the apolipoprotein CIII gene (Hertz et al., 1995). Therefore, it remains possible that ligand-bound TRα is directly repressing transactivation from the AOx-PPRE.

We have shown that the efficient potentiation of the AOx-PPRE by TRα requires the transfection of both PPARα and RXRα. Interestingly, in the absence of transfected RXRα, TRα slightly represses the peroxisome proliferator-dependent transactivation of the AOx-PPRE reporter gene in a dose dependent manner. Therefore, it appears that the availability of RXRα is also a critical determinant in the TRα regulation of the AOx-PPRE. Some groups

have suggested that TRα/RXRα heterodimers are more promiscuous in their DNA binding site preference compared to TRα homodimers (Olson and Koenig, 1997). Although heterodimerization between TRα and PPARα has been reported, however no natural target element for TRα/PPARα heterodimers has been characterized (Bogazzi et al., 1994). TRα associates with the AOx-PPRE as both a monomer and as a TRα/RXRα heterodimer *in vitro*; however, it remains possible that TRα/PPARα heterodimers are influencing the *in vivo* response to peroxisome proliferators. It may be that the monomeric form of TRα is responsible for maintaining a repressive state, whereas unliganded TRα/RXRα augments transactivation by PPARα/RXRα, either through direct interactions with the AOx-PPRE or by other means.

In order to obtain a more precise picture of TRα, PPARα and RXRα interactions with the AOx-PPRE several further experiments would be beneficial. An examination of the roles of shared nuclear receptor accessory factors, such as SMRT and SRC-1 will help to identify the requirements of PPARα, TRα and RXRα for co-repressors and co-activators in the regulation of the AOx-PPRE. Studies which employ mutant TRα will assist in determining a more specific role for this receptor in peroxisome proliferator-signalling. Several TRα mutants have been characterized which exhibit altered capacities for dimerization, ligand binding or association with distinct response elements (Kitajima et al., 1995; Liu et al., 1996; Nagaya et al., 1992). In addition, specific mutations in the ligand binding domains of TRs have been identified which permit differential interactions with co-regulatory factors independent of ligand association (Liu et al., 1998; Nagaya et al., 1998). The use of a yeast system for analysis of nuclear receptor interactions and transactivation has been employed

successfully (Marcus et al., 1996), and may provide a suitable environment in which to further study TR/RXR/PPAR regulation of the AOx-PPRE. A closer investigation of the possible roles of TRα/PPARα heterodimers *in vivo*, the effect of their respective ligands on dimerization and the specific actions of co-regulatory factors will aid in developing a more complete model of TRα, PPARα and RXRα interplay.

6.1.4 RZRα, PPARα and RXRα Interplay

RZRs are one of the few identified nuclear receptors which bind to target response elements as monomers (Carlberg et al., 1994; Giguere et al., 1994). The RZR consensus binding site is conserved within the HD-PPRE and to a lesser extent in the AOx-PPRE. We observed binding of RZRα specifically to the HD-PPRE, and saw potentiation of PPARα transactivation by RZRα from this response element when RXRα levels were limiting. The results of these experiments are summarized in Fig. 6-1. The RZR subfamily of receptors contain a carboxy terminal extension in their DNA-binding domains which is required for the recognition of an extended 3' sequence flanking the core TGACCT-like site (Giguere et al., 1995; McBroom et al., 1995; Wilson et al., 1992). PPARα also contains a similar carboxy terminal extension of its zinc finger which may account for the requirement for 3' sequences flanking PPREs (Hsu et al., 1998). This may also explain why PPARα/RXRα heterodimers exhibit the opposite polarity from most other RXRα heterodimers (Chu et al., 1995a; IJpenberg et al., 1997; Rastinejad et al., 1995). The most commonly observed orientation for RXRα heterodimers is with RXRα binding to the 3' TGACCT-like site (Kurokawa et al., 1994; Rastinejad et al., 1995).

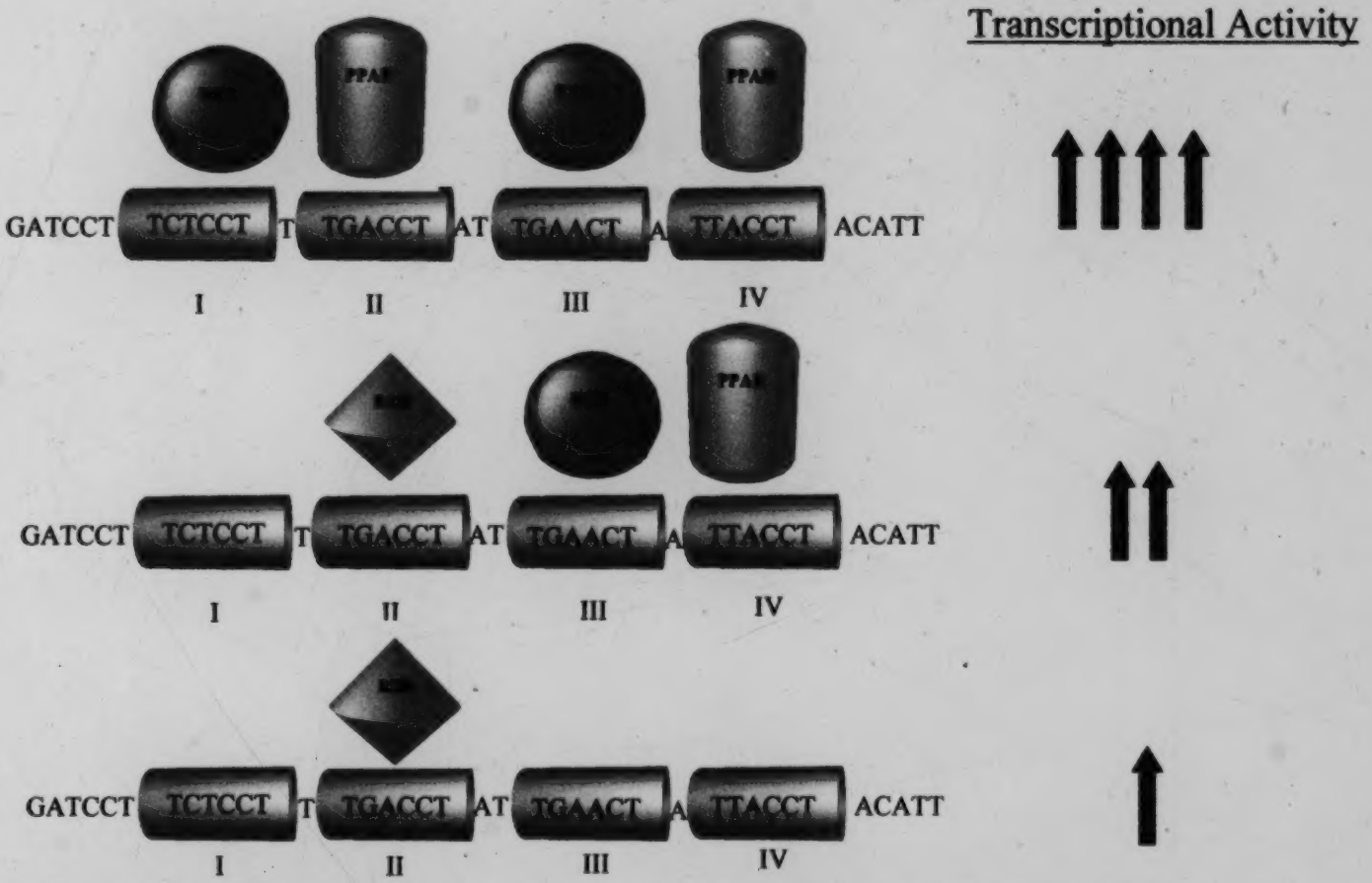


Figure 6-1. Summary of results from studies of RZRα effects on peroxisome proliferator-mediated transactivation from the HD-PPRE.

The model I have provided in Figure 6-2 details the potential interactions by which RZRα may be influencing PPARα-mediated gene expression, however the general means through which RZRα is functioning can be extended to explain how cross-talk may be occurring with other nuclear receptors. This model was derived from the observations that the RZRα monomer recognizes the second TGACCT-related repeat within the HD-PPRE (site II) and that peroxisome proliferator-dependent RZRα potentiation of PPARα activity is only observed in conditions where levels of RXRα are limiting. The overall level of PPARα transactivation is greatly increased when exogenous RXRα is included in co-transfections. It may be that under these conditions any potentiation by RZRα is concealed. It has been proposed that *in vivo*, PPARα/RXRα heterodimers bind to each DR1 site within the HD-PPRE, yielding the highest level of transactivation (Chu et al., 1995a). In conditions of RXRα excess *in vivo*, this conformation may preclude association of RZRα with site II. Based on our *in vitro* DNA-binding studies with HD-PPRE mutants, it appears that PPARα/RXRα heterodimer binding is much stronger at sites III and IV. PPARα/RXRα heterodimers may require additional components that are not present in our in vitro system, to enable binding to sites I and II of the HD-PPRE.

The intermediate level of PPARα-directed transactivation provided by RZRα in conditions of limiting RXRα may be accounted for by several non-mutually exclusive means (see Fig. 6-2). It may be that for the RZRα monomer, association at site II augments PPARα/RXRα transactivation by stabilizing the activated PPARα/RXRα heterodimer bound at sites III and IV, and may help to maintain PPARα/RXRα in a ligand associated conformation. Since RZRα binds to the HD-PPRE at a site directly beside RXRα, it would

Figure 6-2. Possible roles for RZRα in directing peroxisome proliferator-mediatiated transactivation from the HD-PPRE. Arrows indicate putative sites of interaction for the RZRα monomer. RZRα may be altering transcription by (i) stabilizing activated PPARα/RXRα heterodimers, (ii) facilitating assembly/association of a co-activator complex or (iii) assisting dissociation of a co-repressor complex.

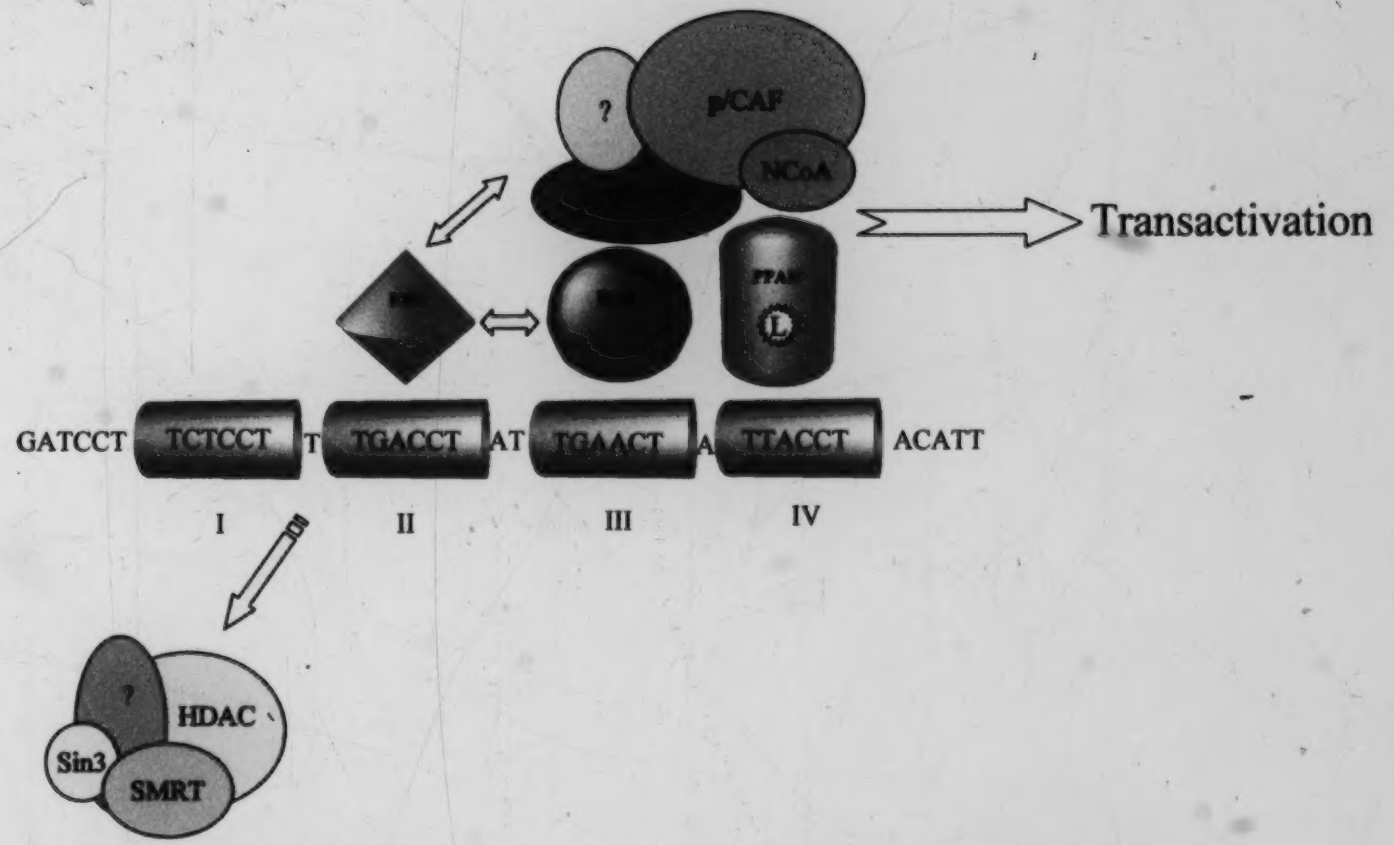


Figure 6-2. Possible roles for RZRα in directing peroxisome proliferator-mediated transactivation from the HD-PPRE.

be interesting to determine if RZRα interacts weakly with aspects of RXRα and to examine what effect the RXRα ligand, 9-*cis*-retinoic acid may have on interactions with RZRα. It does not appear that RZRα is affecting transactivation indirectly, since mutation of the site II eliminates the potentiation of PPARα/RXRα transactivation by RZRα.

The slight repressive effect of RZRα in the absence of a peroxisome proliferator indicates that it is specifically the interactions between ligand activated PPARα/RXRα and RZRα which increased activation from the HD-PPRE, hinting at the importance of PPARα/RXRα/RZRα-co-regulator interactions. Yeast two-hybrid analysis may be of use to identify RZRα-interacting factors and would help to determine what role this nuclear receptor is playing in the assembly of a co-activator complex. RZRα does contain an AF-2 region which is necessary for interaction with known co-activators, however the association of RZRs with co-activators has not yet been examined. Additionally, RZRα may be interacting with p300/CBP or p/CAF or other components of the co-activator complex. RZRα may also assist the displacement of a co-repressor and prevent association of PPARα/RXRα heterodimers with components of a co-repressor complex. It will be interesting to determine if regions of the RZRα monomer interact directly with RXR and/or with PPAR/RXR co-activators or co-repressors.

The conclusive identification of an RZRα ligand will also open the doors for future studies. Although melatonin has been proposed to function as an RZR agonist, we saw no effects of melatonin on RZRα activity in our experiments. Recent evidence has indicated that several thiazolidenediones act as RZR ligands, and this suggests additional possibilities for examining the effects of RZRα on PPAR-directed gene expression (Missbach et al., 1996).

All transfections that we performed employed depleted serum, however it remains possible that an endogenous RZRα ligand is present in our transfection system and therefore RZRα may be already present in an active form.

6.2 General Model of Nuclear Receptor Interactions with the AOx- and HD-PPREs

The regulation of the AOx- and HD-PPREs by nuclear receptors is dynamic and the general modulation of target gene expression by nuclear receptors is by nature a fluid arrangement, allowing rapid and concerted transcriptional responses to appropriate physiological stimuli. A model of nuclear receptor interplay involved in the regulation of the AOx- and HD-PPREs is emerging based on studies in our laboratory and elsewhere (see Fig. 6-3).

The means by which nuclear receptors cross-talk are both direct and indirect (Katzenellenbogen et al., 1996). Nuclear receptors influence transcription directly through competition for binding sites, sequestration of common heterodimerization partners, such as RXRα, and by mediating interactions with co-activators or co-repressors. The effects of nuclear receptor cross-talk may be indirect, through changes in upstream signals, producing physical modifications in downstream receptors or altering ligand availability.

We observed that nuclear receptor competition for shared DNA binding sites occurs with both the AOx- and HD-PPREs. This competition was seen for the AOx-PPRE with PPARα, RXRα, COUP-TF, HNF-4 and TRα through both *in vitro* binding studies and in transient transfections (Marcus et al., 1996; Miyata et al., 1993; Winrow et al., 1996; Winrow et al., 1994). HNF-4 and COUP-TF were found to compete with PPARα/RXRα

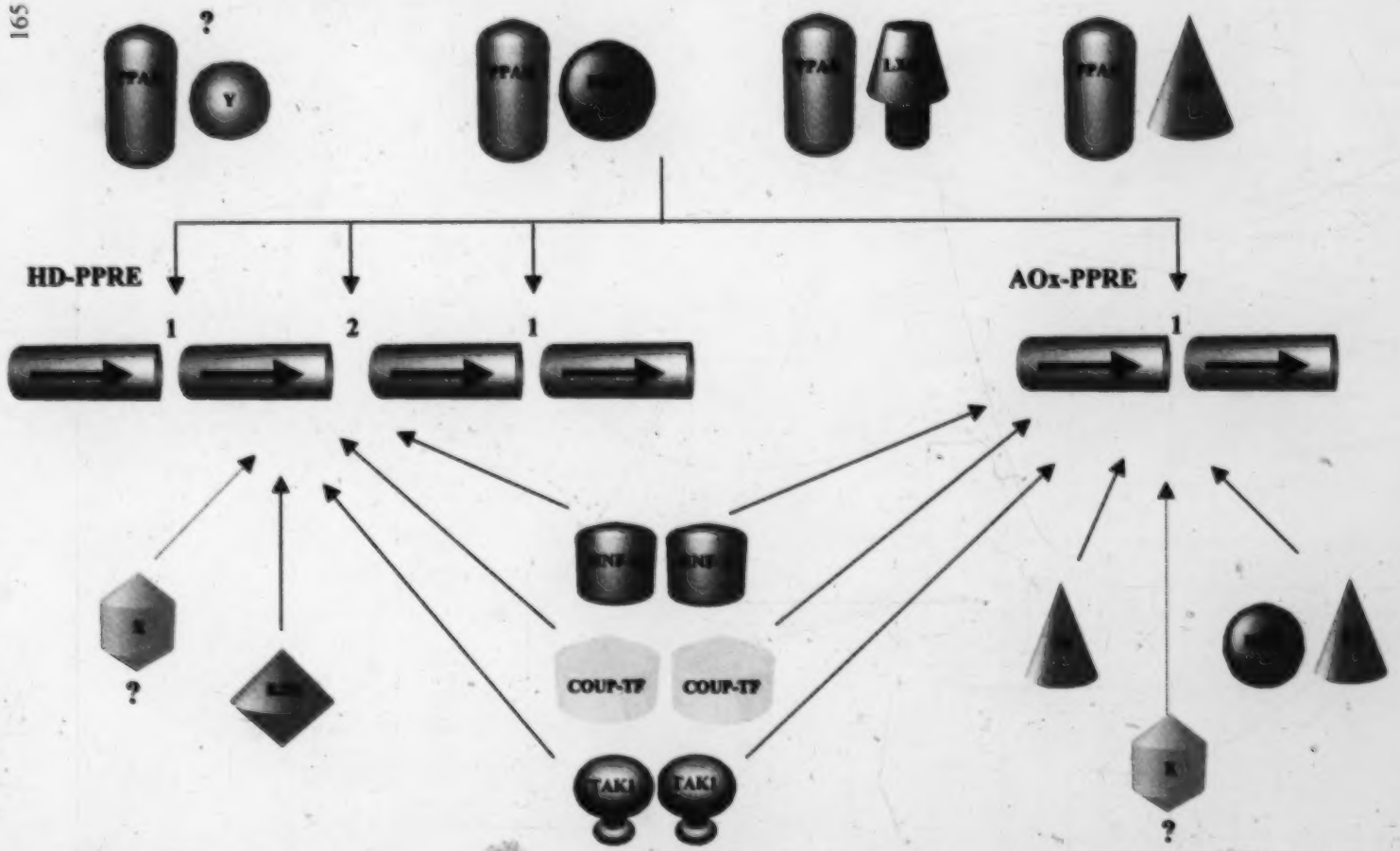


Figure 6-3. Overview of nuclear receptor crosstalk in the transactivation from the AOx- and HD-PPREs.

heterodimers for binding to the HD-PPRE *in vitro* (Marcus et al., 1996; Miyata et al., 1993; Winrow et al., 1994). By virtue of experiments with mutated HD-PPREs, it is becoming apparent that although sites III and IV are important for PPARα/RXRα binding, site II is necessary for the co-regulatory effects of several nuclear receptors (Marcus et al., 1996; Miyata et al., 1993; Winrow et al., 1998; Winrow et al., 1994). Mutations of sites I or II have minimal effects on PPARα/RXRα DNA binding *in vitro* (see Chapter 5, Fig. 5-6) (Miyata et al., 1993). The mutation of site II yields a reduced level of overall activity but is still transactivated by PPARα/RXRα in a peroxisome proliferator-dependent manner in our transfection system (see Chapter 5, Fig. 5-7). Site II is required for the *in vitro* binding of HNF-4, COUP-TF and RZRα and was also shown to be necessary for the PPARα-dependent potentiation of HD-PPRE by RZRα (Miyata et al., 1993; Winrow et al., 1998; Winrow et al., 1994). It is interesting to note that based on the reported polarity of PPARα/RXRα heterodimers, RXRα binds upstream to site III and PPARα binds downstream to site IV (see Fig.6-2) (Chu et al., 1995a; IJpenberg et al., 1997; Kurokawa et al., 1994). Given the importance of site II in the co-regulation of the HD-PPRE, it will be interesting to determine if, for HNF-4 and RZRα, the proximity to RXRα facilitates physical inter-receptor contact, which thereby modifies transactivation. Insight into which domains are necessary for these interactions and how association of 9-*cis*-retinoic acid may influence this interplay will be beneficial to our overall understanding of the co-regulatory mechanisms involved.

TAK1 is a novel orphan nuclear receptor found in hepatocytes, that represses PPARα transactivation from both the AOx - and HD-PPREs (Yan et al., 1998). TAK1 functions as a homodimer and does not interact with either PPARα or RXRα. It was determined that

TAK1 inhibits PPARα/RXRα transactivation by both competing for binding to these PPREs and by sequestering the PPAR co-activator RIP140 (Yan et al., 1998). Similar mechanisms are likely involved in the interplay between PPAR, RXR and other co-regulatory nuclear receptors. In particular, our results suggest that the cross-talk between TRα, PPARα and RXRα involves competition for DNA binding and titration of shared co-activators and co-repressors.

RZRα binds target response elements as a monomer and HNF-4 functions as a homodimer, however TRα can bind the AOx-PPRE as both a monomer and as a heterodimer with RXRα. Competition for a common heterodimeric partner such as RXRα is another means by which TRα may cross-talk with PPARα in regulating the AOx-PPRE. Nuclear receptors may affect PPARα/RXRα transactivation by sequestering either PPARα or RXRα as a heterodimer which will not associate with either the AOx- or HD-PPRE (Miyata et al., 1996). This may be the case for TRα, where TRα/RXRα and TRα/PPARα heterodimers may not recognize the AOx-PPRE *in vivo*. Liver X receptor (LXR) is an orphan nuclear receptor which was identified as a PPARα-interacting factor by a yeast two-hybrid screen (Miyata et al., 1996). LXRα decreases PPARα/RXRα transactivation from the AOx- and HD-PPREs but does not appear to bind to these response elements (Miyata et al., 1996). It was determined that LXRα may be affecting peroxisome proliferator transactivation of the AOx- and HD-PPREs by sequestering PPARα or RXRα, through the formation of LXRα heterodimers which do not recognize these response elements (Miyata et al., 1996).

The presence of a peroxisome proliferator served to augment PPARα/RXRα-dependent transactivation from the AOx-PPRE by TRα and from the HD-PPRE by RZRα and

HNF-4. This may be a result of direct stabilization of the active PPARα/RXRα heterodimer, possibly by maintaining PPARα in an agonist bound state. Additionally, this potentiation may be occurring through the titration of a co-repressor, thereby preventing assembly of a co-repressor complex and sustaining the chromatin in an acetylated conformation. The positive transcriptional effects of TRα, HNF-4 and RZRα may also be manifested by facilitating interactions of PPARα/RXRα with a co-activator or assisting assembly of a co-activator complex. PPARα, RXRα and TRα associate with common co-activators, and it is probable that HNF-4 and RZRα also share a number of identical co-activator molecules.

HNF-4, RZRα and TRα could affect peroxisome proliferator modulated transactivation indirectly by modifying the upstream signalling of the peroxisome proliferator pathway. It has been reported that PPARγ is a phosphoprotein that is phosphorylated on serine residues through the mitogen-activated protein kinase cascade (Adams et al., 1997; Camp and Tafuri, 1997). It is possible that HNF-4, RZRα and/or TRα might modulate the phosphorylation states of PPARs or RXRα by indirectly altering the activity of a required kinase. Alternatively, these nuclear receptors may affect some other step of peroxisome proliferator signal transduction, such as ligand availability, and thereby alter PPARα/RXRα-mediated gene expression. This can be easily envisioned in the case of PPAR where ligands include prostaglandins, fatty acids and leukotrienes whose synthesis or modification may very well occur as a result of pathways directed by TRα, HNF-4 or RZRα or other nuclear receptors. The recent observation that HNF-4 activity is modulated by binding of fatty acyl-CoA thioesters is particularly suggestive of ligand directed interplay with PPARα, given that many long chain fatty acids function as peroxisome proliferators (Dreyer et al., 1993; Forman

et al., 1997a; Hertz et al., 1998; Kliewer et al., 1997; Krey et al., 1997; Willson and Wahli, 1997).

We have demonstrated that calreticulin, which modulates cellular Ca^{2+}-storage and signalling, can dramatically alter the transactivation potential of the GR, while exerting no discernible influence on PPARα/RXRα signalling. It is thought that although calreticulin recognizes a consensus amino acid motif found in the DNA binding domains of virtually all nuclear receptors, the effect of calreticulin on receptor activity may be indirect and possibly due to a combination of alterations of cellular Ca^{2+} levels and endoplasmic reticulum signalling (Baksh et al., 1995; Krause and Michalak, 1997; Mery et al., 1996; Michalak et al., 1996).

6.3 Summary

Peroxisomes are the site of multiple processes including bile acid and cholesterol biosynthesis, degradation of H_2O_2 and β-oxidation of fatty acids. The regulation of gene expression by nuclear receptors is a dynamic process which provides for transcriptional responses to a multitude of signalling pathways. I have examined the interplay between nuclear receptors in the transcriptional regulation of genes involved in peroxisomal β-oxidation. In addition I have investigated the influence of a Ca^{2+}-regulatory protein, calreticulin, which has been shown to affect the activity of several nuclear receptors, in PPAR-mediated transcriptional activation.

HNF-4 binds target response elements as a homodimer and was shown to bind readily to the AOx-PPRE and to a lesser extent to the HD-PPRE. In the absence of a peroxisome

proliferator, HNF-4 inhibited transactivation by PPARα/RXRα heterodimers from both the AOx- and HD-PPREs. HNF-4 differentially regulated transactivation by PPARα/RXRα when a peroxisome proliferator was included in co-transfections. Under these conditions PPARα/RXRα transactivation from the AOx-PPRE was inhibited by HNF-4, whereas activation of the HD-PPRE was increased by HNF-4. This differential effect of HNF-4 could be a reflection of differences in DNA response element affinity of the receptor and may involve additional interactions with common nuclear receptor accessory factors.

Calreticulin was shown to interact *in vitro* with a number of nuclear receptors via a conserved amino acid motif in their DNA binding domains. Although calreticulin inhibited transactivation by GR, no effect of calreticulin on the activity of PPARα/RXRα heterodimers was observed in a whole cell setting. We demonstrated that calreticulin is not a general regulator of nuclear receptor activity, as had been proposed. It is most likely that the effect of calreticulin on gene expression is indirect, possibly due to alterations in intracellular Ca^{2+} levels which might influence responsive nuclear receptors.

Unliganded TRα is able to bind to the AOx-PPRE *in vitro* and potentiates transactivation of this element by peroxisome proliferator-stimulated PPARα in a cellular context. Addition of the TR ligand, T3, potently repressed PPARα-mediated transcriptional activity for the AOx-PPRE when TRα was present. Although TRα is able to bind to the AOx-PPRE as both a monomer and as a TRα/RXRα heterodimer, it is possible that unliganded TRα potentiates PPARα activity by sequestration of a common co-repressor.

RZRα binds specifically to the HD-PPRE as a monomer but does not bind to the AOx-PPRE. The binding of the RZRα monomer was localized to the second TGACCT-like

repeat within the HD-PPRE, and this site was shown to be essential for RZRα potentiation of PPARα activity *in vivo*. In conditions where RXRα was limiting, RZRα augmented the activity of PPARα from the HD-PPRE in an agonist-dependent manner. It appears that RZRα is mediating this effect by binding to the HD-PPRE at a site adjacent to RXRα and possibly stabilizing the ligand-bound form of the PPARα/RXRα complex, inhibiting association with a co-repressor and/or facilitating assembly of a co-activator complex.

These findings show how the PPAR, RXR, HNF-4, TR and RZR signalling pathways are interconnected in the synergistic or differential expression of the AOx and HD genes by virtue of their PPREs. The intricate communication between diverse networks of nuclear receptors enables a high degree of specificity and confers the ability to rapidly respond to diverse physiological stimuli, be they transient or long-term. This research extends the current knowledge regarding peroxisome proliferator stimulation of gene expression and conveys the complexity of interactions involved in the coordinate regulation of the peroxisomal β-oxidation pathway at the level of transcription.

6.4 Conclusions

The research presented herein illustrates the integration of multiple signalling networks in the concerted regulation of genes encoding the first two enzymes of the peroxisomal β-oxidation cascade. Taken together these findings demonstrate the multilevel control of the AOx- and HD-PPRES through cross-talk between diverse nuclear receptor signalling pathways. The observations obtained, provide insight into the specific mechanisms of gene regulation by PPARα and RXRα, and at the same time indicate several directions for

future studies which will prove fruitful in expanding our knowledge of the general mechanisms through which the effects of nuclear receptors are mediated. The implications of this research touch not only the transcriptional regulation of genes responsible for peroxisomal β-oxidation, but also underscore the overall importance of nuclear receptor interplay and afford firm indications as to the co-regulatory roles of distinct nuclear receptors.

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